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Serum-Derived Plasminogen Is Activated by Apoptotic Cells and Promotes Their Phagocytic Clearance

Matthias Rosenwald,*† Uwe Koppe,*† Hildegard Keppeler,* Guido Sauer,‡ Roman Hennel,§ Anne Ernst,§ Karin Erika Blume,* Christoph Peter,*‡ Martin Herrmann,§ Claus Belka,§ Klaus Schulze-Osthoff,§ Sebastian Wesselborg,*‡ and Kirsten Lauber*†

The elimination of apoptotic cells, called efferocytosis, is fundamentally important for tissue homeostasis and prevents the onset of inflammation and autoimmunity. Serum proteins are known to assist in this complex process. In the current study, we performed a multistep chromatographic fractionation of human serum and identified plasminogen, a protein involved in fibrinolysis, wound healing, and tissue remodeling, as a novel serum-derived factor promoting apoptotic cell removal. Even at levels significantly lower than its serum concentration, purified plasminogen strongly enhanced apoptotic prey cell internalization by macrophages. Plasminogen acted mainly on prey cells, whereas on macrophages no enhancement of the engulfment process was observed. We further demonstrate that the efferocytosis-promoting activity essentially required the proteolytic activation of plasminogen and was completely abrogated by the urokinase plasminogen activator inhibitor-1 and serine protease inhibitor aprotinin. Thus, our study assigns a new function to plasminogen and plasmin in apoptotic cell clearance. **The Journal of Immunology, 2012, 189: 5722–5728.***

The present study was designed to identify serum proteins promoting apoptotic cell engulfment. By employing a multistep chromatographic fractionation of human serum and mass spectrometry, we identified plasminogen as a novel factor stimulating corpse engulfment.

**Materials and Methods**

**Cells and reagents**

Primary human macrophages were generated by differentiation of positively selected (anti-CD14 magnetic beads; Miltenyi Biotec, Bergisch Gladbach, Germany) peripheral monocytes with 20 ng/ml M-CSF or GM-CSF (both from R&D Systems, Heidelberg, Germany), respectively, in macrophage medium (Invitrogen Life Technologies, Heidelberg, Germany) supplemented with 5% autologous serum for 7 d. Human neutrophils were obtained by double-Ficoll gradient purification (Histopaque 1.119 g/ml, Sigma-Aldrich, Deisenhofen, Germany; Ficoll-Paque 1.077 g/ml, GE Healthcare, Freiburg, Germany) and used as prey cells 24 h after preparation, when they had undergone spontaneous apoptosis.

THP-1 cells were differentiated into macrophages with 10 nM PMA (Sigma-Aldrich) for 2.5 d, irradiated with 10 mJ/cm² UV-C to undergo apoptosis, or heated to 58°C for 20 min to induce necrosis, respectively (7, 8). Plasminogen, plasma, plasmin, and plasminogen activator inhibitor-1 (PAI-1) were purchased from American Diagnostica (Heidelberg, Germany), plasminogen-FITC from Biomac (Leipzig, Germany), and plasmin-Cy2 was prepared with the Cy2-labeling kit (GE Healthcare), according to the manufacturer’s instructions. Recombinant human plasmin (produced in yeast) was obtained from Merck Millipore (Darmstadt, Germany). Recombinant human Glu-plasminogen was expressed and purified from an Escherichia coli BL21(DE3) expression clone carrying the cDNA encoding human Glu-plasminogen in pET15b (Merck Calbiochem). The his-tagged protein was purified on a Ni-NTA His-Bind resin column (Merck Calbiochem) and used as prey cells 24 h after preparation, when they had undergone spontaneous apoptosis.

Chromatographic fractionation of human AB serum

All chromatography steps were carried out on a BioLogic FPLC Workstation (Bio-Rad, Munich, Germany) with resin or prepacked columns from GE Healthcare. Human AB serum was subjected to native ammonium sulfate precipitation (80% saturation). The resulting protein pellet was resolved in 10 ml 20 mM Tris-HCl (pH 7.4) and 160 mM NaCl and desalted on a HiPrep 26/10 desalting column. The protein fraction of the flow-through was collected at OD280nm >2.0. For removal of IgG, the desalted protein pool (600 mg protein) was passed over a protein G Sepharose column (3.5 ml gel bed). After elution of bound IgG at pH 4.0 with 20 mM Tris-HCl (pH 7.4), the flow-through was collected at OD280nm >0.2. Protein concentrations were determined using a BioRad protein assay (BioRad, Munich, Germany) and an extinction coefficient at OD280nm of 1.4 (26). Deproteinized sera were dialyzed against 20 mM Tris-HCl (pH 7.4) and stored at −80°C. Human AB serum was subjected to native ammonium sulfate precipitation (80% saturation). The resulting protein pellet was resolved in 10 ml 20 mM Tris-HCl (pH 7.4) and 160 mM NaCl and desalted on a HiPrep 26/10 desalting column. The protein fraction of the flow-through was collected at OD280nm >2.0. For removal of IgG, the desalted protein pool (600 mg protein) was passed over a protein G Sepharose column (3.5 ml gel bed). After elution of bound IgG at pH 4.0 with 20 mM Tris-HCl (pH 7.4), the flow-through was collected at OD280nm >0.2. Protein concentrations were determined using a BioRad protein assay (BioRad, Munich, Germany) and an extinction coefficient at OD280nm of 1.4 (26). Deproteinized sera were dialyzed against 20 mM Tris-HCl (pH 7.4) and stored at −80°C.

**Abbreviations used in this article:** PAI, plasminogen activator inhibitor; PI, propi-
2.6 and re-equilibration of the column, this step was repeated to a total of four times. To reduce the albumin content of the sample, the protein G flow-through (400 mg protein) was passed over a Cibacron Blue affinity column (5 ml Hitrap Blue). The flow-through was collected and combined with the bound nonalbumin proteins eluted at 500 mM NaCl in 20 mM Tris-HCl (pH 7.4). After elution of albumin with 2 M NaCl in 20 mM Tris-HCl (pH 7.4) and re-equilibration of the column, this step was repeated with the flow-through fraction to a total of three runs.

The IgG-depleted, albumin-reduced protein pool (90 mg protein in 20 mM bis-Tris-HCl [pH 6.0]) was subjected to cation exchange chromatography on a Mono S HR 5/5 Sepharose column (1 ml gel bed). After washing with 5 column volumes, elution was performed by applying an ascending linear salt gradient (0–800 mM NaCl in 20 mM bis-Tris-HCl [pH 6.0]) over 20 column volumes. Fractions of 500 μl were collected and analyzed for phagocytosis-promoting activity. The most active peak fractions were pooled and adjusted to 20 mM Tris-HCl (pH 7.4) in 1 M ammonium sulfate for hydrophobic interaction chromatography.

The protein pool obtained in the Mono S separation step was further fractionated on a HiTrap phenyl-Sepharose column (1 ml gel bed). After washing with 5 column volumes, elution was carried out by applying a descending linear salt gradient (1–0 M ammonium sulfate) over 20 column volumes to the final elution buffer (20 mM Tris-HCl [pH 7.4], 10% glycerol). Fractions of 500 μl were collected and analyzed for their phagocytosis-promoting activity. The most active fractions were pooled on the basis of the OD280 nm profile and concentrated on MultiScreen Ultracel-10 Filter Plates (MWCO 10 kDa; Millipore) against serum-free culture medium.

Mass spectrometry
After SDS-PAGE and silver-staining protein, bands of interests were excised, destained, and in-gel digested with trypsin (Promega, Heidelberg, Germany) following a published protocol (9). Extracted peptides were desalted with self-made microcolumns (10) and separated by reversed-phase HPLC (nanoLC2D; Eksigent, Dublin, CA) using a capillary column with 15 cm length and 75 μm i.d. SilicaTip (PicoTip; New Objective, Woburn, MA) self packed with ReproSil-Pur C18-AQ, 3 μm (Maisch, Ammerbuch-Entringen, Germany). Mass spectrometric analysis was performed on an ion trap (HCTultra; Bruker Daltonics, Bremen, Germany) equipped with a nanoESI source from Proxeon Biosystems (Odense, Denmark).

Mascot generic data files were created using DataAnalysis (Bruker) and searched against the SwissProt protein database (V54.2) using Mascot Server (V2.2). The following settings were used: digestion with trypsin allowing 1 miss cleavage, carbamylation of cysteine as fixed modification, oxidation of methionine as variable, 0.3-Da peptide, and fragmentation mass accuracy.

Flow cytometric measurement of phosphatidylserine exposure, plasminogen, and plasmin binding
Phosphatidylserine (PS) exposure was flow cytometrically measured by annexin V FITC/propidium iodide (PI) staining (annexin V-FLUOS staining kit; Roche, Penzberg, Germany). Cells with positive annexin V FITC, but negative PI signal were considered apoptotic, whereas cells double positive for annexin V FITC and PI staining were considered necrotic (7).

To analyze surface binding, plasminogen FITC or plasmin Cy2 (10 μg/ml) was incubated with 1 × 106 cells in serum-free medium for 20 min at room temperature. Afterward, cells were collected by centrifugation, resuspended in serum-free medium supplemented with 2 μg/ml PL, and subjected to FACS analysis.

Phagocytosis assay
Phagocytosis was measured, as described previously (7,11). Briefly, 1 × 107 prey cells/ml were labeled with 2 μM PKH26 in diluent C (Sigma-Aldrich) for 5 min at room temperature, according to the manufacturer’s instructions. After extensive washing, prey cells were irradiated with 10 mJ/cm2 UV-C for 5 min at room temperature, according to the manufacturer’s instructions. After trypsinization and analysis by flow cytometry, Phagocytosis was measured as the percentage of macrophages with internalized prey cells (double positive for PKH67 and PKH26 staining) on the basis of all macrophages deployed (positive for PKH67 staining).

SDS-PAGE, Coomassie staining, silver staining, and immunoblot analyses
SDS-PAGE, Coomassie, silver staining, and immunoblot analyses were performed, as described previously (12). Anti-human poly(ADP-ribose) polymerase Ab was obtained from Enzo Life Sciences (Lörrach, Germany), and anti-human caspase-3 Ab was from BD Biosciences (Heidelberg, Germany).

Plasmin activity test
A total of 5 × 10⁵ cells was incubated with plasminogen in serum-free medium at 37°C for 2 h. Cell-free supernatants were collected and assessed for plasmin activity with 100 μM fluorogenic peptide substrate ALK-amc (Fremont, CA). The increase in fluorescence was monitored at 37°C over 1 h. Plasmin activity was calculated from the baseline-corrected slope of the curves and is given as FU/min.

Depletion of plasminogen from human AB serum
Plasminogen was specifically depleted from human AB serum via lys- Sepharose chromatography, according to the manufacturer’s instruction (GE Healthcare). Removal of plasminogen was monitored by SDS-PAGE with subsequent Coomassie staining and quantitative analysis on a Licor Odyssey Fluorescence Imaging System.

FIGURE 1. Serum-derived factors promote apoptotic cell engulfment. (A) Human AB serum enhances the phagocytosis of apoptotic cells by macrophages in a dose-dependent manner. Apoptotic, PKH26-labeled THP-1 monocytes were incubated with PMA-differentiated, PKH67-labeled THP-1 macrophages in a 2:1 ratio for 2 h in the presence of 0–10% (v/v) serum, before phagocytosis of prey cells was assessed by flow cytometry. (B) Engulfment-enhancing effect of serum at different macrophage/prey ratios. ***p < 0.01, unpaired two-tailed Student t test analysis of serum-free versus serum-containing samples at the respective preyn/macrophage ratios. (C) Serum-derived phagocytosis-enhancing factors act mainly on the prey cells. Macrophages or prey cells were separately preincubated with 5% (v/v) serum for 30 min. Cells were washed and phagocytosis was analyzed in parallel in samples with and without serum addition. **p < 0.01, unpaired two-tailed Student t test. (D) Complement and noncomplement factors contribute to the serum-derived phagocytosis-enhancing effect. Human AB serum was left untreated (native) or was decomplemented by heat treatment (56°C, 30 min). Subsequently, the serum samples were added at a final concentration of 5% (v/v) to a phagocytosis assay. ***p < 0.01, unpaired two-tailed Student t test.
Data analyses and statistics

If not stated otherwise, results are displayed as mean values ± SD of intra-assay replicates of one representative of at least three independent experiments. The *p* values were calculated by heteroskedastic, unpaired, two-tailed Student *t* test, and asterisks indicate *p* < 0.01 (**) or *p* < 0.05 (*), respectively.

Results

Addition of human serum augments the engulfment of apoptotic cells

Several serum proteins, including complement proteins and protein S, have been described to play a role in apoptotic cell clearance (3–5, 13). We examined in depth the influence of human serum on apoptotic cell engulfment as measured by a flow cytometric phagocytosis assay (Supplemental Fig. 1A–D) and observed that serum enhanced the phagocytic uptake of apoptotic cells in a dose-dependent manner (Fig. 1A, Supplemental Fig. 2A). This effect was clearly detected at different macrophage/prey cell ratios in phagocytosis assays employing PMA-differentiated THP-1 macrophages engulfing apoptotic THP-1 monocytes or primary human macrophages engulfing autologous apoptotic neutrophils, respectively (Fig. 1B, Supplemental Fig. 2B). Moreover, serum pre-incubation of either macrophages or prey cells revealed that the serum effect on phagocytosis targets prey cells rather than macrophages (Fig. 1C). Complement inactivation (heat treatment with 56˚C for 20 min) partially attenuated the phagocytosis-enhancing effect, but heat-treated serum still significantly promoted apoptotic cell engulfment (Fig. 1D).

Chromatographic fractionation of the phagocytosis-promoting factors in human AB serum

To identify the responsible serum factor(s), we performed a multi-step chromatographic separation of human serum (Fig. 2A). The protein extract obtained by ammonium sulfate precipitation was IgG depleted and reduced in albumin by affinity chromatography. The resulting protein fraction was subjected to a two-step separation strategy. (A) Flow chart illustrating the fractionation strategy. (B) Mono S cation exchange chromatogram. Fractionation of the IgG-depleted, albumin-reduced protein fraction of human AB serum (90 mg protein) was performed. Elution was monitored on the basis of OD$_{280}$nm and conductivity. Fractions with highest phagocytosis-enhancing activity (gray area) were combined and further purified by hydrophobic interaction chromatography. (C) Phenyl-Sepharose chromatogram. The active fraction obtained after Mono S chromatography (500 µg protein) was loaded on a phenyl-Sepharose column. Elution was monitored on the basis of OD$_{280}$nm and conductivity. Fractions with highest phagocytosis-enhancing activity (gray area) were pooled and concentrated by ultrafiltration. The gray-shaded protein fraction was analyzed by SDS-PAGE and subsequent mass spectrometry. (D) SDS-PAGE of the serum fraction enriched for prophagocytic activity following cation exchange and hydrophobic interaction chromatography. After SDS-PAGE, proteins were visualized by silver staining and identified by mass spectrometry. Most abundant proteins are depicted. Plasminogen was identified with sequence coverage of 47% to be one of the major components.
using cation exchange and hydrophobic interaction chromatography (Fig. 2B, 2C). Fractions with the highest phagocytic activity from each purification step were collected. Subsequent SDS-PAGE revealed four major protein bands that were analyzed by mass spectrometry. Apart from albumin and fragments of the complement proteins C3 and C4, plasminogen was unambiguously identified (Fig. 2D). It should be noted that protein S, a serum protein with known effecrocytosis-promoting activity, was found in a different fraction pool than plasminogen (Fig. 2D).

**Plasminogen crucially contributes to the phagocytosis-promoting effect of serum**

To characterize the role of plasminogen in apoptotic cell removal, we used purified plasminogen and observed that it dose dependently stimulated the uptake of apoptotic, but not viable cells by PMA-differentiated THP-1 macrophages as well as M-CSF- or GM-CSF-differentiated primary human macrophages (Fig. 3A, Supplemental Fig. 2C). Similar to protein S, plasminogen promoted phagocytosis even at doses significantly lower than its serum concentration (Fig. 3A). Notably, plasminogen did not alter PS exposure on apoptotic cells (data not shown). The purity of commercially purchased plasminogen was confirmed by SDS-PAGE, Coomassie, and silver staining (Supplemental Fig. 3A, 3B). However, to exclude that nondetectable contaminants in purified plasma-derived plasminogen contributed to the measured enhancement of apoptotic cell engulfment, we also tested purified recombinant human Glu-plasminogen and observed a comparable increase in phagocytosis as in the case of plasma-derived plasminogen (Supplemental Fig. 3C).

Intriguingly, plasminogen-depleted serum stimulated apoptotic cell uptake significantly less efficiently than the same serum reconstituted with plasminogen or native serum, respectively (Fig. 3B, 3C). Similar to complete serum (Fig. 1C), plasminogen-dependent engulfment enhancement was exerted primarily on prey cells, but not on macrophages (Fig. 4A). However, we did not detect enhanced binding of FITC-labeled plasminogen to apoptotic cells in comparison with viable cells, whereas necrotic cells strongly bound plasminogen (Fig. 4B). Hence, the plasminogen-mediated effect on

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**FIGURE 3.** Plasminogen crucially contributes to the phagocytosis-promoting effect of serum. (A) Purified plasminogen promotes the internalization of apoptotic, but not viable cells. Phagocytosis of viable and apoptotic THP-1 monocytes by PMA-differentiated THP-1 macrophages was assessed in the absence or presence of the indicated serum concentrations of plasminogen. Protein S and kininogen (an unrelated serum protein) served as positive and negative controls, respectively. The 100% serum concentration correlates to 120 µg/ml plasminogen, 25 µg/ml protein S, and 120 µg/ml kininogen. *p < 0.05 and **p < 0.01 (respectively) in unpaired two-tailed Student t test analysis of plasminogen- or protein S–containing samples versus the serum-free control. (B) Specific depletion of plasminogen from human serum. AB serum was subjected to lys-Sepharose chromatography, and the removal of plasminogen was monitored by SDS-PAGE, Coomassie staining (right panel), and quantitative fluorescence analysis of the individual lanes (left panel). The lys-Sepharose eluate served to identify the plasminogen band in the serum samples. Reconstitution was performed with 120 µg/ml purified plasminogen. (C) Plasminogen-depleted serum displays a profoundly weaker phagocytosis-promoting effect. Native, plasminogen-depleted, and plasminogen-reconstituted serum were applied to a phagocytosis assay, as in Fig. 1A. Differences in phagocytosis in comparison with the serum-free control are displayed. *p < 0.05 and **p < 0.01 (respectively) in unpaired two-tailed Student t test.
Efferocytosis was not due to opsonization or coating. Apart from opsonization, plasminogen might support corpse engulfment by its zymogen protease activity. To address this issue, viable and apoptotic cells were incubated with plasminogen, and subsequently, plasmin activity was measured. Intriguingly, although apoptotic and viable cells did not differ in plasminogen binding, a profound activation of plasminogen by apoptotic cells was observed (Fig. 4C). We next examined whether the proteolytic activity of plasmin is required for its phagocytosis-promoting effect. As shown in Fig. 4D, the serine protease inhibitor aprotinin and PAI-1 efficiently inhibited plasminogen-dependent phagocytosis. These findings suggest that plasmin is the active component, which was further substantiated by the observation that purified plasmin—plasma derived as well as recombinant human plasmin—stimulated phagocytosis even at amounts corresponding to 0.1–1% of its zymogen serum concentration (Fig. 5A, Supplemental Fig. 3C). Nevertheless, plasmin—like plasminogen—did not exhibit an enhanced binding to apoptotic cells (Fig. 5B). In contrast, thrombin, a protease whose zymogen form is present in serum concentrations similar to plasminogen, displayed no phagocytosis enhancement.

Finally, we addressed the question, in which phase of apoptosis plasmin(ogen) exerts its efferocytosis-promoting effect. To this end, we employed prey cells in different stages of the apoptotic progress as measured by PS exposure, caspase, and caspase-substrate processing (Supplemental Fig. 1E, 1F). Notably, these cells had not transited into secondary necrosis, as was confirmed by PI exclusion staining. When applied in a phagocytosis assay, we detected no plasminogen-mediated efferocytosis enhancement in the case of the early apoptotic cells 4 h after apoptosis induction (Fig. 6). The onset of plasminogen-mediated efferocytosis enhancement was observed with prey cells 8 h after apoptosis induction (when PS exposure had reached its saturation level), passed through a maximum with prey cells 12 h after apoptosis induction, and declined again with prey cells 16 h after apoptosis induction. These data suggest that plasmin (ogen) can only support the engulfment of apoptosing cells, which readily expose “eat-me” signals, such as externalized PS.
By employing a multistep chromatographic fractionation of human serum, we have identified plasminogen as a novel factor that promotes the phagocytic removal of apoptotic cells. Serum concentrations of plasminogen are $\sim 120 \, \mu g/ml$, which is $\sim 10$-fold higher than the concentration required to stimulate efferocytosis. Furthermore, we have shown that in this context the proteolytic activation/activity of plasmin(ogen) is of crucial importance. The question that arises is how plasminogen exerts its effect on corpse clearance. Previous reports have described an enhanced binding of plasminogen to the surface of apoptotic cells, suggesting that it might function as an opsonin (14–17). In the current study, however, we did not observe enhanced surface binding of plasminogen to apoptotic cells. This might be explained by the conditions or cell types used. For instance, in previous reports, enhanced plasminogen binding paralleled the loss of plasma membrane integrity as a late event of apoptosis, whereas our analyses included only early, apoptotic cells with an intact plasma membrane. Instead of acting as an opsonin, we show that plasminogen enhances efferocytosis under crucial contribution of its proteolytic activity, which is acquired after interaction with apoptotic cells. Our data are in line with studies from other groups that have attributed this activation of plasminogen to an increased expression of urokinase-type plasminogen activator, which was specifically detected on the surface of apoptotic, but not necrotic cells (15–17). Notably, plasmin(ogen)-mediated enhancement of dying cell engulfment was observed in a phase of apoptosis, in which the maximum level of PS externalization had already been reached and the integrity of the plasma membrane was still intact, suggesting that the exposure of “eat-me” signals is a prerequisite for plasmin(ogen)-dependent phagocytosis promotion.

The mechanisms responsible for plasmin(ogen)’s phagocytic effect remain elusive. Nevertheless, plasmin’s cleavage specificity is limited to lysine and arginine residues, and interestingly, gingipain R and clostripain, bacterial proteases with cleavage specificity after arginine, have been described to enhance apoptotic cell phagocytosis as well (18). Therefore, similar substrates might be cleaved by these proteases. One such putative target was proposed to be the “don’t-eat-me” signal CD31 (19), because CD31 surface staining declined after gingipain and clostripain treatment (18). Whether a “don’t-eat-me” signal is indeed degraded or a novel “eat-me” signal is generated by the bacterial proteases and plasmin(ogen) remains to be investigated.

Overall, we have identified a novel and hitherto undescribed activity for plasmin(ogen) in apoptotic cell clearance. Notably, the urokinase plasminogen activator receptor has recently been described as a novel engulfment receptor (20), although its phagocytic role remains controversial (21). Moreover, Das and Plow (22) have described the histone 2B-dependent recruitment of plasminogen.
to PS-rich surfaces, and it has been shown that PAI impairs apoptotic cell removal (23). These studies taken together with our work suggest that dysregulation of the plasminogen system might lead to deranged apoptotic cell clearance. Of note, plasminogen-deficient mice display delayed involution of the mammary glands (24, 25). This process is closely linked to apoptotic cell removal and comparably impaired in mice lacking the engulfment factor MFG-E8 (26). Further studies will help to elucidate whether plasminogen deficiency is associated with defects in corpse clearance and the onset of chronic inflammation and autoimmunity.

Disclosures
The authors have no financial conflicts of interest.

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