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J Immunol 2014; 192:3540-3547; Prepublished online 10 March 2014; doi: 10.4049/jimmunol.1302864
http://www.jimmunol.org/content/192/8/3540

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/07/jimmunol.1302864.DCSupplemental

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Urokinase Plasminogen Activator Is a Central Regulator of Macrophage Three-Dimensional Invasion, Matrix Degradation, and Adhesion

Andrew J. Fleetwood,* Adrian Achuthan,† Heidi Schultz,** Anneline Nansen,‡ Kasper Almholt,§ Pernille Usher,† and John A. Hamilton*

Urokinase plasminogen activator (uPA) and its receptor (uPAR) coordinate a plasmin-mediated proteolytic cascade that has been implicated in cell adhesion, cell motility, and matrix breakdown, for example, during inflammation. As part of their function during inflammatory responses, macrophages move through tissues and encounter both two-dimensional (2D) surfaces and more complex three-dimensional (3D) interstitial matrices. Based on approaches employing uPA–gene–deficient macrophages, plasminogen supplementation, and neutralization with specific protease inhibitors, it is reported in this study that uPA activity is a central component of the invasion of macrophages through a 3D Matrigel barrier; it also has a nonredundant role in macrophage-mediated matrix degradation. For murine macrophages, matrix metalloproteinase-9 activity was found to be required for these uPA-mediated effects. Evidence for a unique role for uPA in the inverse relationship between macrophage adhesion and 2D migration was also noted: macrophage adhesion to vitronectin was enhanced by uPA and blocked by plasminogen activator inhibitor-1, the latter approach also able to enhance in turn the 2D migration on this matrix protein. It is therefore proposed that uPA can have a key role in the inflammatory response at several levels as a central regulator of macrophage 3D invasion, matrix remodeling, and adhesion. The Journal of Immunology, 2014, 192: 3540–3547.

Received for publication October 24, 2013. Accepted for publication February 6, 2014.

This work was supported by grants and a senior principal research fellowship (to J.A.H.) from the National Health and Medical Research Council of Australia and by Novo Nordisk A/S.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 2D, two-dimensional; 3D, three-dimensional; BMM, bone marrow–derived macrophage; ECM, extracellular matrix; MDM, monocyte-derived macrophage; MMP, matrix metalloproteinase; MMP9−/−, MMP9–gene–deficient; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase plasminogen activator; Thio-Mg, thio-glycollate-elicited peritoneal macrophage; uPA−/−, uPA–gene–deficient; uPAR, uPA receptor; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302864
and uPA levels in the synovium of rheumatoid arthritis patients correlate with disease severity (35–39). In addition, we and others demonstrated a dependence on uPA for arthritis development in three systemic mouse models (40, 41), supporting the concept that uPA is a major player in inflammatory arthritis (36–38, 42). Whether the proteolytic cascade initiated by uPA is central to macrophage infiltration into and/or efflux out of inflamed tissues (such as the rheumatoid arthritis synovium) remains to be seen.

As for leukocytes in general, important functions of macrophages are their migratory activity and interactions in intact tissues. Among leukocytes, macrophages have the highest expression of uPA (12), with expression levels increasing as cells mature from monocytes into macrophages (43, 44). Because the uPA/αPAR system has been implicated in cellular adhesion and detachment (14, 23, 27), the role of uPA in macrophage 2D migration in vitro has been addressed but with contradictory findings (45, 46); because uPA/plasmin-mediated proteolysis has also been implicated in matrix remodeling (9–13), a detailed analysis of the role of uPA in macrophage motility (i.e., 3D invasion and 2D migration) is clearly warranted. We report in this study that uPA proteolytic activity is required for optimal macrophage 3D invasion through a Matrigel barrier and for matrix degradation. uPA also promotes macrophage adhesion to the ECM, thereby impairing their 2D migration. Thus, uPA can act at several levels during an inflammatory response as a central regulator of macrophage 3D invasion, matrix remodeling, and adhesion.

Materials and Methods

Mice

Female C57BL/6 mice (8–12 wk) were supplied by Monash University (Clayton, VIC, Australia) (47). The uPA gene–deficient mice (uPA−/−), provided by Dr. P. Carmeliet (University of Leuven, Leuven, Belgium), were backcrossed onto the C57BL/6 background for 11 generations. The MMP9 gene–deficient (MMP9−/−) mice were a gift from Steven Shapiro (Washington University) and Prof. Zena Werb (University of California, San Francisco) and backcrossed onto a BALB/c background by Dr. Normand Pouliot and Associate Prof. Robin Anderson at the Peter MacCallum Cancer Centre (East Melbourne, VIC, Australia) (48). All experiments were approved by the Royal Melbourne Hospital Research Foundation Animal Ethics Committee.

Reagents

Reagents were as follows: recombinant human M-CSF (CSF-1; Chiron) (49), MCP-1 (R&D Systems), IMLF (Sigma-Aldrich), Plg (Enzyme Research Lab), aprotinin (Sigma-Aldrich), PAI-1 (Molecular Innovations), α2-antiplasmin (Innovative Research), vitronectin (Sigma-Aldrich), and GM6001 (Millipore).

Preparation of mouse bone marrow–derived macrophages and human monocyte-derived macrophages

Mouse bone marrow–derived macrophages (BMM) and human monocyte-derived macrophages (MDM) were prepared as before (50). Briefly, bone marrow cells from the femurs of mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM GlutaMax-I, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of M-CSF (CSF-1; 2000 U/ml). On day 7, adherent cells were harvested. Human monocytes were purified from buffy coats (Red Cross Blood Bank, Melbourne, VIC, Australia) and MDM generated as before from M-CSF–treated cultures (50). Peritoneal exudate cells were elicited by i.p. injection of 1 ml Brewer’s thioglycollate medium (Difco), and thioglycollate-elicited peritoneal macrophages (Thio-Mφ) were harvested 4 d later as previously described (51).

Migration (2D) and invasion (3D) assays

Macrophages (1 × 10^5 in 200 μl RPMI + 10% FCS) were loaded into the upper chamber of 24-transwell (BD Biosciences; 8 μm) inserts, uncoated or coated with vitronectin (10 μg/well), in the case of 2D migration assays, or coated with 100 μl Matrigel (BD Biosciences; according to the manufacturer’s instructions) in the case of 3D invasion assays. For the purposes of this paper, we have adopted the nomenclature used by others (52, 53) for the description of migration assays on transwells as 2D migration assays. In some experiments, Plg (250 nM), α2-antiplasmin (300 nM), PAI-1 (30 nM), GM6001 (5 μM), or aprotinin (10 nM) were added to upper chambers. A total of 750 μl RPMI (plus 10% FCS) supplemented with M-CSF (2000 U/ml), MCP-1 (100 nM), or IMLF (200 nM) was placed in the lower chamber to provide a chemotactrant gradient. Macrophage 2D migration was measured at 24 h. 3D invasion was measured at 4 h for BMM and Thio-Mφ or at 24 h for MDM. These were optimal time points determined by kinetic analysis of 2D migration and 3D invasion. At completion of the assay, cells in the upper chamber were removed and the adherent cells in the lower chamber stained (DiffQuik; Bio-Scientific) and quantified by counting cells in five random fields (at original magnification ×20) for each filter. All cells (BMM, Thio-Mφ, and MDM) in the lower chamber were attached to the lower surface of the transwell chamber. The assays were performed in triplicate in four independent experiments.

Matrix degradation assay

Macrophage matrix degradation was determined using a Gelatin Invadopodia Assay (Millipore). Briefly, coverslips were coated with FITC-coupled gelatin and macrophages (3 × 10^4) cultured on the gelatin for 48 h in the presence of M-CSF (1000 U/ml), then fixed with 4% paraformaldehyde, processed for F-actin and DAPI staining, and visualized by fluorescent microscopy (Zeiss Axioskop 2; Carl Zeiss; at original magnification ×20). In some experiments, matrix degradation was measured in the presence of Plg (250 nM), α2-antiplasmin (300 nM), PAI-1 (30 nM), GM6001 (5 μM), or aprotinin (10 nM). Images were captured with a Zeiss AxioCam MRm (Carl Zeiss) and quantification of matrix degradation assessed by calculating the area of gelatin degradation per total cell area by image analysis software (ImageJ, version 1.46; National Institutes of Health) (54). Three random fields (at original magnification ×20) were analyzed for each group, and the assays were performed in three independent experiments.

Adhesion assay

To perform adhesion assays, 96-well microtiter plates (Costar, Cambridge, MA) were left uncoated or coated with 100 μl vitronectin (10 μg/ml) for 18 h at 4˚C. After blocking the wells for 2 h in 2% BSA, macrophages (2 × 10^5 cells/well) in RPMI 1640 (10% FCS) were added to each well and allowed to adhere for 1 h at 37˚C. In some experiments, macrophage adhesion was measured in the presence of PAI-1 (Molecular Innovations). At the completion of the assay, the nonadherent cells were removed and wells washed gently three times with PBS. The number of adherent cells remaining was determined by using CellTiter-Glo (Promega) reagent that is bioreduced into colored formazan measured at 490 nm. The assays were performed in triplicate in four independent experiments.

Statistical analysis

Statistical comparisons between groups were performed using unpaired Student t test (GraphPad Prism 4 software; GraphPad). The p values ≤0.05 indicate significance. Data were plotted using GraphPad Prism 4.03 software (GraphPad).

Results

uPA proteolytic activity is required for optimal macrophage 3D invasion through a matrix barrier

Macrophage 3D invasion through Matrigel has previously been shown to be inhibited by a mixture of protease inhibitors (7). However, even though there has been speculation, the identity of the specific protease(s) involved in such invasion has not yet been determined (6, 7). We hypothesized that uPA-mediated proteolysis might be crucial to macrophage 3D invasion and matrix turnover. Firstly, the ability of BMM and thioglycollate-elicited peritoneal macrophages (Thio-Mφ) from wild-type (WT) and uPA−/− mice to invade through a 3D matrix of Matrigel was tested. We found that significantly fewer uPA−/− BMM and uPA−/− Thio-Mφ (Fig. 1A) were capable of 3D invasion through Matrigel relative to WT cells. These data suggest that uPA has a crucial role in promoting macrophage 3D invasion.

To provide additional evidence for this concept, we next tested the requirement for uPA proteolytic activity. Firstly, we seeded WT BMM onto the Matrigel layer in the presence of inhibitors of uPA and plasmin proteolytic activities. We found that α2-antiplasmin...
(the major plasmin inhibitor), PAI-1 (a major inhibitor of uPA), and aprotinin (the broad-spectrum serine protease inhibitor) all potently reduced BMM 3D invasion (Fig. 1B). These findings most likely reflect the ability of these modulators to inhibit the activation by uPA of endogenous Plg present in the cultures, possibly originating from the serum. Secondly, further supporting a role for uPA in the proteolytic events promoting 3D invasion, we found that BMM 3D invasion was greatly potentiated by the addition of Plg to the upper chamber with approximately twice the number of BMM invading (Fig. 1B). a2-antiplasmin, PAI-1, and aprotinin again potently blocked this Plg-enhanced BMM invasion. Thirdly, 3D invasion assays were also performed with uPA−/− BMM, in the absence and presence of exogenous Plg. The increase of 3D invasion by Plg was completely abolished when uPA−/− BMM were tested (Fig. 1B). All of these data together indicate that uPA proteolytic activity is required for optimal mouse macrophage 3D invasion through Matrigel.

We also addressed the role of the PA/Plg system in the 3D invasion of human macrophages. For MDM invasion through Matrigel toward M-CSF, a2-antiplasmin, PAI-1, and aprotinin all tended to reduce MDM invasion through Matrigel in the absence of exogenous Plg (Fig. 1C), although, unlike in the murine system (Fig. 1B), these differences did not reach statistical significance. However, we again found that addition of Plg enhanced invasion by ~3-fold and that this Plg-enhanced invasion could once more be blocked by a2-antiplasmin, PAI-1, and aprotinin (Fig. 1C).

**uPA-mediated proteolysis is required for macrophage matrix degradation**

Given the likely link with matrix invasion, we next hypothesized that uPA would also be a central component of the proteolytic pathway, leading to macrophage-mediated matrix degradation. To address this question, BMM were seeded on gelatin-FITC–coated coverslips in the presence of Plg, a2-antiplasmin, PAI-1, or aprotinin and matrix degradation quantified by fluorescent microscopy. As can be seen in Fig. 2A, untreated BMM had a relatively low capacity to degrade gelatin-FITC, which could be dramatically increased by the addition of Plg (Fig. 2C for quantification), thus correlating with the ability of Plg to enhance BMM 3D invasion (Fig. 1B). In further agreement with our 3D invasion data, we found that a2-antiplasmin, PAI-1, and aprotinin were all capable of significantly blocking the Plg-induced gelatin-FITC degradation by BMM (Fig. 2A, 2C). As further evidence of a requirement for uPA in the proteolytic breakdown of this ECM-like material, we found no detectable gelatin-FITC degradation by uPA−/− BMM and that these cells did not respond to Plg for increased matrix degradation (Fig. 2B, 2C for quantification).

In similar experiments using human macrophages, we found that Plg significantly increased gelatin-FITC degradation by MDM.

**FIGURE 1.** uPA proteolytic activity is required for optimal macrophage 3D invasion through a matrix barrier. (A) BMM and Thio-M6 (WT and uPA−/−) were placed in the upper chamber of transwell inserts that were coated with Matrigel. M-CSF (2000 U/ml) and fMLF (200 nM) were placed in the lower chamber as indicated and macrophage invasion (4 h) was quantitated (see Materials and Methods). BMM (WT and uPA−/−) (B) or MDM (C) were placed in the upper chamber of transwell inserts that were coated with Matrigel. a2-antiplasmin (a2-AP) (300 nM), PAI-1 (30 nM), aprotinin (Apro) (10 nM), or Plg (250 nM) were added to the upper chambers as indicated. M-CSF (2000 U/ml) was placed in the lower chamber and BMM (4 h) and MDM (24 h) invasion quantitated (see Materials and Methods). Data are expressed as mean number of cells per field ± SEM (n = 4). *p ≤ 0.05 (Student t test). −, untreated.

**FIGURE 2.** uPA proteolytic activity is required for BMM matrix degradation. BMM (WT; 3 × 104 cells/well) (A) and BMM (uPA−/−; 3 × 104 cells/ well) (B) were placed on FITC-coupled gelatin-coated coverslips and incubated for 48 h, either untreated (−) or in the presence of Plg (250 nM) ± a2-antiplasmin (a2-AP; 300 nM), PAI-1 (30 nM), or aprotinin (Apro; 10 nM) as indicated. Cells were fixed and stained with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (top panel, red) and DAPI (top panel, blue), and gelatin (bottom panel) degradation was visualized in areas devoid of FITC staining by fluorescent microscopy at original magnification ×20 (see Materials and Methods). (C) Quantification of gelatin-FITC degradation by BMM (WT and uPA−/−). The area of gelatin degradation per total cell area was determined and expressed as a percentage ± SEM relative to untreated (−) BMM (WT or uPA−/−). Shown are data from a representative of three independent experiments. *p ≤ 0.05 (Student t test).
MMP9 activation is linked to uPA/Plg-mediated 3D invasion and matrix degradation by murine macrophages

uPA-catalyzed conversion of Plg to plasmin is the beginning of a complex proteolytic cascade that also activates other ECM-degrading proteinases, such as MMP3, MMP9, and MMP12 (55). To address whether MMP9 was specifically involved in macrophage 3D invasion, we generated BMM from MMP9−/− mice and tested their ability to invade through a 3D Matrigel matrix. As can be seen in Fig. 4A, we found that MMP9−/− BMM had a significantly impaired ability to invade through Matrigel relative to WT BMM. Furthermore, the Plg-induced increase of BMM 3D invasion was completely dependent on the presence of MMP9, with Plg having no effect on the 3D invasion of MMP9−/− BMM (Fig. 4A).

We also found that the broad-spectrum MMP inhibitor GM6001 blocked WT BMM 3D invasion, both in the absence and presence of Plg (Fig. 4A). These data suggest a connection between uPA/Plg and MMP9 in murine macrophage 3D invasion.

To test whether MMPs were involved in human macrophage 3D invasion, MDM invasion toward M-CSF was also measured in the presence of GM6001. Similar to above (Fig. 1C), we found that Plg again increased MDM 3D invasion but, in contrast to BMM, GM6001 had no impact on MDM invasion in the absence (data not shown) or presence of Plg (Fig. 4B).

To test whether uPA/Plg and MMP9 could also be linked in macrophage matrix degradation, we measured the ability of MMP9−/− BMM to degrade a gelatin-FITC matrix. As can be seen in Fig. 4C, MMP9−/− BMM were unable to do so and addition of Plg failed to enhance matrix degradation (Fig. 4D for quantification), which was in stark contrast to our earlier observation with WT BMM (Fig. 2A, 2C) but similar to our findings with uPA−/− BMM (Fig. 2B). These data show that Plg-induced increase of BMM 3D invasion (Fig. 4A) and matrix degradation (Fig. 4C) were both dependent on the presence of MMP9. Contrarily, we found that GM6001 was unable to inhibit MDM matrix degradation (Fig. 4E, 4F for quantification), consistent with its inability to block MDM 3D invasion (Fig. 4B).

These data show that MMP9 is implicated in the uPA/Plg-mediated induction of BMM 3D invasion and matrix degradation, suggesting that MMP9 activation is an important downstream component of the proteolytic pathway(s) activated by uPA in murine macrophages. In contrast, in the human system, MDM 3D invasion and matrix degradation, which can be blocked by inhibitors of the PA system (Figs. 1C, 3A), appear to be independent of MMPs and other metalloproteases sensitive to GM6001.
uPA-induced adhesion to the ECM impairs macrophage 2D migration

Studies on uPA involvement in 2D migration have largely been restricted to human PBMCs (45) and ill-defined murine bone marrow precursor cells (46) with different outcomes. In this study, we analyzed the role of uPA in regulating macrophage 2D migration (56) in transwell chambers that were left uncoated or coated with vitronectin. For 2D migration of WT and uPA−/−BMM through uncoated wells, when M-CSF, MCP-1, and fMLF were placed in the lower chambers as chemotactic agents, it was found in each case that uPA−/−BMM had significantly increased migration compared with WT BMM (2- to 3-fold after 24 h; Fig. 5A). The increased migration in the absence of uPA was also seen for uPA−/− versus WT Thio-Mø (Fig. 5A). Interestingly, in contrast to 3D invasion, BMM 2D migration was not affected by the addition of Plg or α2-antiplasmin to the upper chamber (data not shown), suggesting 2D migration is independent of plasmin-mediated proteolysis. Together, these data indicate that macrophage uPA impairs 2D migration.

Cellular migration requires coordination of adhesion and detachment to the ECM (8), with the extent of migration dependent on the avidity of adhesion (57). The binding of uPA to uPAR is known to promote cell adhesion to vitronectin, leading to complexes between uPAR and a range of integrins (20–24). Given its ability to promote cellular adhesion, we speculated that uPA-mediated adhesion may be contributing to the reduced macrophage 2D migration by the WT cells. To test this, we first measured the adhesion of uPA−/−BMM and WT BMM to microtiter plates that were uncoated (plastic) or coated with vitronectin. Adhesion was measured after 1 h using CellTiter-Glo labeling. uPA−/−BMM were significantly less adherent to vitronectin compared with WT BMM (Fig. 5B). uPA−/−BMM adhesion also tended to be lower on uncoated surfaces (Fig. 5B), consistent with their more rounded appearance compared with the classic spindle-shaped morphology of their WT counterparts in culture (Fig. 5C, 5D).

To further elucidate the role of uPA in the inverse relationship between macrophage adhesion and 2D migration (8), we determined the effect of PAI-1 on murine and human macrophage adhesion. PAI-1 has been shown to inhibit adhesion of certain cell lines to vitronectin (14, 31). PAI-1 dose-dependently decreased the adhesion of both BMM (Fig. 6A) and human MDM (Fig. 6B) to vitronectin-coated surfaces. As a control for any potential non-specific effects of PAI-1 in decreasing WT BMM adherence (Fig. 6A), we found that PAI-1 did not affect adhesion of uPA−/−BMM (data not shown). The decreased adhesion to the uncoated (plastic) surface was not dose dependent over the same dose range, with the maximal inhibition obtained at the lowest PAI-1 concentration (1 nM) possibly being sufficient to inhibit the adhesion induced by the vitronectin present in the serum-containing medium. These data are consistent with the uPA−/−macrophage data (Fig. 5B), supporting the notion that uPA promotes macrophage adhesion to the ECM.

Given the link between uPA/Plg and MMP9 in regulating BMM 3D invasion and matrix degradation, we sought to address whether MMP9 was specifically involved in macrophage adhesion and 2D migration. Adhesion of MMP9−/−BMM to plastic and vitronectin-coated surfaces was similar to WT BMM (Supplemental Fig. 1A), and MMP9−/−BMM had no defect in 2D migration on vitronectin-coated transwells (Supplemental Fig. 1B). These data suggest that MMP9 is not involved in macrophage adhesion and 2D migration.

![FIGURE 5.](http://www.jimmunol.org/) uPA impairs macrophage 2D migration but promotes adhesion. (A) BMM and Thio-Mø (WT and uPA−/−) were placed in the upper chamber of transwell inserts that were uncoated (2D migration). M-CSF (2000 U/ml), MCP-1 (100 nM), and fMLF (200 nM) were placed in the lower chamber as indicated, and macrophage migration (24 h) was quantitated (see Materials and Methods). Data are expressed as mean number of cells per field ± SEM (n = 4). (B) BMM (WT and uPA−/−) adhesion to uncoated and vitronectin-coated (VTN; 10 μg/ml) microtiter plates was determined after 1 h. At the completion of the assay, the nonadherent cells were removed, and the number of adherent macrophages remaining was determined by addition of CellTiter-Glo (see Materials and Methods). Data are expressed as mean MTS absorbance at 490 nm (A490nm) ± SEM (n = 4). Morphology of WT BMM (C) and uPA−/−BMM (D) at day 7 in culture (original magnification ×20). Arrows indicate the classic spindle-shaped macrophage morphology. Shown are data from a representative of three independent experiments. *p ≤ 0.05 (Student t test).

![FIGURE 6.](http://www.jimmunol.org/) uPA-induced adhesion to the ECM impairs macrophage 2D migration. BMM (WT) (A) and MDM (B) adhesion to uncoated and vitronectin (VTN)-coated (10 μg/ml) microtiter plates was determined in the presence of PAI-1 for 1 h. At the completion of the assay, the nonadherent cells were removed, and the number of adherent macrophages remaining was determined by addition of CellTiter-Glo (see Materials and Methods). Data are expressed as mean MTS absorbance at 490 nm (A490nm) ± SEM (n = 4). BMM (WT and uPA−/−) (C) and MDM (D) were placed in the upper chamber of transwell inserts that were uncoated or vitronectin-coated (10 μg/ml) PAI-1 (30 nM) was added to upper chamber. M-CSF (2000 U/ml) was placed in the lower chamber, and macrophage migration (24 h) was quantified (see Materials and Methods). Data are expressed as mean number of cells per field ± SEM (n = 4). *p ≤ 0.05 (Student t test). −, untreated.
We next determined whether PAI-1 inhibition of macrophage adhesion to a vitronectin-coated surface influenced their migration on vitronectin. The migration of WT BMM was significantly reduced when the top surface of the transwell was coated with vitronectin (Fig. 6C). This reduction of 2D migration by vitronectin could be reversed when PAI-1 was added at concentrations shown earlier to reduce macrophage adhesion (Fig. 6A). Contrarily, the heightened uPA−/− BMM 2D migration (Fig. 5A) was not affected by the addition of vitronectin or PAI-1 (Fig. 6C). Similarly, MDM migration was reduced on vitronectin-coated transwells, which could also be abrogated by the addition of PAI-1 (Fig. 6D). Together, these data suggest that vitronectin-induced adhesion leads to a reduction in macrophage 2D migration that is dependent on uPA (Fig. 7).

**Discussion**

In systemic models of inflammatory arthritis, we and others have previously demonstrated a deleterious role for uPA (40, 41). We found that uPA−/− mice were resistant to disease with reduced cellular infiltration into the joints (41). The reason(s) for the proarthritogenic action of uPA in these models is unclear, although we (38, 40, 41) and others (36, 37) have speculated that there may be effects on cell migration and tissue destruction. Given these background data, we examined above the role of uPA in the regulation of macrophage motility and matrix degradation.

Because most of the data concerning the role of uPA in monocyte/macrophage 3D invasion has come from studies of cell lines (58–60) or investigations focused on Plg biology (58, 60), we specifically addressed uPA biology in primary macrophage populations. The data shown in this study indicate for in vitro and ex vivo mouse macrophages, and most likely for the human MDM, that uPA contributes profoundly both to optimal matrix degradation and also to 3D invasion (the formal proof that it is uPA that is responsible for our findings with the human macrophages will require specific neutralization, for example, by Abs). These effects of uPA appear to be dependent on its proteolytic activity given that inhibitors, viz. PAI-1, α2-antiplasmin, and aprotinin, significantly abrogate invasion and matrix degradation, whereas exogenous Plg enhanced them. The conversion of Plg to active plasmin appeared to be dependent on uPA activity (and not tissue-type PA) given the inability of uPA−/− BMM to respond to exogenous Plg by way of invasion. These data suggest that, for BMM at least, uPA, and not tissue-type PA, is the PA involved in the generation of plasmin in these assay systems. We therefore propose that uPA may be the unidentified macrophage (non-MMP) protease that contributes to the pseudopod-linked mesenchymal mode of migration (i.e., invasion through a 3D matrix) (7). Whether uPA is involved in macrophage 3D invasion through other more biologically relevant matrices, such as fibrillar versus gelled collagen, is currently under investigation.

As regards to the mechanism, we found that MMP9 generation was a crucial component of the downstream proteolytic pathway activated by uPA in BMM. Evidence for a link between uPA and MMP generation in monocytes/macrophages has been previously presented (61, 62). We showed above that MMP9−/− and uPA−/− BMM adopt very similar phenotypes, with both populations being poorly invasive and unable to degrade a gelatin matrix. Also, in the absence of MMP9, macrophages were completely unable to respond to Plg for increased 3D invasion and matrix degradation, indicating its importance in the downstream proteolytic events initiated by uPA/Plg. Consistent with our data, Thio-M₈ in the presence of Plg generate active MMP9 (63), and if these macrophages are elicited from MMP9−/− mice, they fail to respond to Plg for increased 3D invasion (64). In that study, MMP9−/− Thio-M₈ had no defect in 3D invasion, which differs from our finding with MMP9−/− BMM and with uPA−/− Thio-M₈. We found that human macrophage 3D invasion and matrix degradation were enhanced by Plg but did not involve MMP activity, the last observation paralleling previous work (7). These data suggest that uPA/Plg-mediated proteolysis is central to macrophage 3D invasion and matrix degradation but the specific downstream proteolytic strategies employed are cell and/or species specific.

The effects of uPA in the current study were not limited to regulation of 3D invasion and matrix degradation. We demonstrated that macrophage adhesion to vitronectin could be promoted by uPA and blocked by its inhibitor, PAI-1. This inhibition of uPA-mediated ECM adhesion by PAI-1 was associated with an increased 2D migration, which is consistent with data showing that PAI-1 abrogates the formation of adhesive interactions with vitronectin, leading to enhanced cellular migration (14). PAI-1 may detach cells from vitronectin by binding to the uPA present in uPA–uPAR–integrin complexes (23, 27) or by directly binding vitronectin in competition with uPAR (31). Which of these mechanisms is occurring in our assay system is currently being addressed as is the ability of uPA (and uPAR) to regulate macrophage adhesion and 2D migration on ECM proteins other than vitronectin. These data underscore the often inverse relationship between adhesion and migration (65, 66) and the importance of uPA in these processes (Fig. 7).

Our studies indicate that uPA impairs macrophage 2D migration in response to a range of chemotactic stimuli. Loss of uPA has previously been found to have different effects on migration; uPA promoted 2D migration toward MLP and MCP-1 after 90 min in a study using an immature bone marrow precursor population (46). A likely explanation for this difference is that our studies were conducted in the presence of serum, whereas previous studies were conducted under serum-free conditions (67). Serum contains a significant amount of vitronectin (68), providing a substrate for uPAR-dependent adhesion. The presence of uPA under these conditions likely reduces migration by promoting adhesion to the substratrum.

![FIGURE 7](http://www.jimmunol.org/)
We and others (40, 41) have previously provided evidence that targeting uPA in systemic models of inflammatory arthritis provides protection from disease, possibly through effects on cell migration and tissue destruction (40, 41). Increased macrophage numbers in the rheumatoid arthritis synovium correlate with poor outcomes (2, 69), and successful diverse therapies all result in reduced synovial macrophage cellularity (1, 3). Based on the evidence above, we propose the model in Fig. 7, in which uPA is a central regulator of macrophage adhesion, matrix degradation, and tissue invasion. In the model, uPA-enhanced macrophage adhesion to a tissue matrix would increase the retention time, thus allowing for uPA proteolytic activity, via Plg, to degrade, directly or indirectly, the matrix, thereby also enabling the cell to move through the tissue. uPA blockade would prevent this sequence of events, thus helping to explain the benefit of its targeting in inflammatory disease (19, 38, 40, 41, 70).

Acknowledgments

We thank Jennifer Davis for assistance with the maintenance and care of the mice.

Disclosures

A.N., K.A., and P.U. are full-time employees and minor shareholders of Novo Nordisk A/S.

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