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ROLE OF FORMIC RECEPTORS IN SOLUBLE UROKINASE RECEPTOR INDUCED HUMAN VASCULAR SMOOTH MUSCLE MIGRATION

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Abstract

Background—Vascular smooth muscle cell (VSMC) migration in response to urokinase is dependent on binding of the urokinase molecule to the urokinase receptor uPAR and cleavage of the receptor. The aim of this study is to examine the role of the soluble uPAR (suPAR) in vascular smooth muscle cell migration.

Methods—Human VSMCs were cultured in vitro. Linear wound and Boyden microchemotaxis assays of migration were performed in the presence of suPAR. Inhibitors to G-protein signaling and kinase activation were employed to study these pathways. Assays were performed for MAPK and EGFR activation.

Results—suPAR induced concentration-dependent migration of VSMC, which was G protein-dependent and was blocked by G α i and G β γ inhibitors. Removal of the full uPAR molecule by incubation of the cells with a phospholipase did not interfere with this response. suPAR induced ERK1/2, p38^{MAPK} and JNK activation in a G α i/G β γ -dependent manner and interruption of these signaling pathways prevented suPAR-mediated migration. suPAR activity was independent of

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CONTRIBUTIONS:

Enrico A. Duru: concept and design, performance of the work, analysis and/or interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published

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DISCLOSURE

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plasmin activity. suPAR did not activate EGFR. Interruption of the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL1*) but not high affinity N-formyl-Met-Leu-Phe receptor (*FPR*) prevented cell migration and activation in response to suPAR. suPAR increased MMP-2 expression and activity, and this was dependent on the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL1*) and ERK1/2.

Conclusion—suPAR induces human smooth muscle cell activation and migration independent of the full uPAR through activation of the G protein-coupled receptor *FPRL1* which is not linked to the plasminogen activation cascade.

Keywords

uPA; soluble uPAR; migration; cell signaling; human coronary smooth muscle cell

INTRODUCTION

The serine protease, urokinase plasminogen activator (uPA) is the primary serine protease (plasminogen activator) involved in vessel remodeling processes (1) and increased serum uPA is associated with development of restenosis after coronary angioplasty (2). Within the micro-environment of the cell wall, uPA can also be cleaved by other proteases into several biologically active fragments: aminoterminal fragment (ATF), kringle domain (K) and carboxyterminal fragment (CTF). Each appears to have unique characteristics (3–5). We have shown that ATF will induce plasmin-independent cell migration and that CTF will induce plasmin-dependent cell proliferation (3). Urokinase requires binding of its aminoterminal domain to uPAR on the cell surface to initiate migration. Urokinase, through its protease domain is also capable of cleaving the cell surface receptor at its glycosylphosphatidylinositol (GPI) tail. If one uses ATF, which contains only the receptor binding domain of urokinase one can isolate the receptor binding from protease activity. ATF induces plasmin-independent cell migration in vascular smooth muscle cells (VSMCs) (3). This migration requires the binding to the GPI-tethered uPAR receptor. uPAR exists on the membrane in a complex of multiple receptors including Low Density Lipoprotein Receptor-related Protein (LRP), integrins and Epidermal Growth Factor Receptor (EGFR). Our recent data has demonstrated that ATF will induce smooth muscle cell migration through trans-activation of *erbB1* in an A Disintegrin And Metalloproteinase (ADAM)-dependent manner and is Mitogen- Activated Protein Kinases (MAPK) dependent (3, 6, 7). After ATF binds uPAR, there is cleavage of the uPAR receptor into a cell-bound D1 fragment and a soluble D2/D3 fragment, soluble uPAR (suPAR). D2/D3 which has been shown to bind to the low affinity receptor of N-formyl-methionyl-leucyl-phenylalanine (fMLP), formyl peptide receptor-like-1 (*FPRL1*), to induce a G α i-mediated responses (8). Other receptors of the family of FPRL, formyl peptide receptor (FPR) and formyl peptide receptor-like-2 (FPRL2) have also been shown to respond to the same peptide sequence on suPAR and are desensitized by the suPAR chemotactic fragments (9–11). The aim of this study is to test the hypothesis that *FPRL1* is required for suPAR-induced cell signaling and suPAR-mediated cell migration in human VSMC.

METHODS

Experimental Design

Human coronary arterial VSMCs were cultured in vitro (passage 3–6; Clontech, Mountain View, CA). Linear wound and Boyden microchemotaxis assays of migration were performed in the presence of suPAR, ATF or fMLP. Dose response assays for migration and the ED50 for each agonist used. Assays were performed for MAPK and EGFR activation. Chemical and molecular inhibitors to G-protein signaling, EGFR, fMLP receptors and MAPK activation were employed to study these pathways.

Materials

suPAR and ATF were purchased from American Diagnostica, Inc. (Greenwich, CT). fMLP, Pertussis toxin (G α i inhibitor, 100ng/ml), Phosphatidylinositol-specific-phospholipase C (100 μ g/mL), PD98059 (ERK inhibitor, 25 μ M) SB203580 (p38^{MAPK} inhibitor, 10 μ M), SP600125 (JNK inhibitor, 1 μ M), EGFR inhibitor (AG1478, 10 μ M), ϵ -aminocaproic acid (EACA; plasmin inhibitor, 100 μ M), aprotinin (plasmin inhibitor, 100 units/ml) and GM6001 (MMP inhibitor, 10nM) were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated anti-rabbit IgG antibody (raised in goat) and peroxidase-conjugated anti-mouse IgG antibody (raised in goat) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Phospho-ERK1/2 antibody was purchased from Promega, Inc. (Madison, WI). Total ERK 1/2 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Phospho-p38^{MAPK} and phospho-JNK antibodies were purchased from Biosource (Camarillo, CA). Total p38^{MAPK} and JNK antibodies were purchased from Cell Signaling (Beverly, MA). Dulbecco's minimal essential medium (DMEM) and Dulbecco's phosphate buffered saline (dPBS) were purchased from Corning Cellgro (Corning, NY).

Wound Assay

The wound assay was performed with VSMC as previously described (3, 6). Human VSMC were grown to confluence in 60mm² dishes and then starved (1% serum) for 24 hrs in the presence of hydroxyurea (5mM, Sigma Chemical Co) to prevent proliferation. Thereafter, each dish was divided into a 2 $\times$ 3 grid. With the use of a 1–200 μ l pipette tip, a linear wound was made in each hemisphere of the dish. Immediately after wounding, media was changed to fresh DMEM (for all reagent dishes and as negative control) or 10% FBS (positive control). Cells were then allowed to migrate over 24 hours at 37°C in DMEM with or without suPAR (10 nM), ATF (10nM) or fMLP (10nM). In a second series of experiments, migration in response to the agonists was examined in the presence and absence of the inhibitors to G-protein signaling, fMLP receptors and MAPK activation. Under a 40 $\times$ lens with an attached SPOT camera (Diagnostic Instruments, Inc.), images were taken of the intersections of the linear wound and each grid line. This resulted in eight fields per dish. Cells were allowed to migrate over 24 hrs. at 37°C. Each field was measured at time 0 and at 24 hrs. The area of each field was measured using SPOT Advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI), and eight fields from each dish were averaged. Trials with each reagent or inhibitor were performed in six separate dishes, and the results were averaged.

Boyden Chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) and polycarbonate filters (Neuro Probe, Inc., 10 μ m pore size, 25 $\times$ 80 mm, PVP free) with VSMC as previously described (3, 6). suPAR (10 nM), ATF (10 nM), or fMLP (10nM) was added to the lower wells. For trials with the inhibitors, the inhibitor was added to 2 mL of the cell suspension 1 hour prior to addition of cells to the upper wells. Trials included eight or twelve wells per reagent or inhibitor per trial, and were repeated no fewer than three times.

Multiplex Assay

Cells were allowed to grow to 80% confluence and starved (1% serum) for 48hours. Cells were then stimulated with agonist and in the presence of pharmacological and molecular inhibitors. VSMC were harvested at time points from 0 to 30 minutes. The peak activity of the MAPKs was determined from time course experiments and the effects of pharmacological and molecular inhibitors were determined at the same time point. Protein purification was performed using the AllPrep RNA/Protein isolation kit from Qiagen (Valencia, CA). Protein concentrations were determined by the bicinonic acid (BCA) method. Samples were analyzed in a Luminex® 200™ System using 96-well plate using the manufacturer's protocol (Luminex Corporation, Austin, TX) and commercially available assay kits (Millipore Corporation, Billerica, MA).

Western Blotting

Cells were allowed to grow to 80% confluence and starved for 48 hours. Cells were then stimulated with suPAR (10 nM) alone and in the presence of pharmacological and molecular inhibitors and harvested at time points from 0 to 30 minutes. Western blotting was performed as previously described (12). Total protein was determined using antibodies against each intact kinase.

Adenoviral Infection

Adenoviral vectors were constructed by Welgen, Inc. (Worcester, MA) using purified plasmid encoding β ARK_{CT}, obtained from Guthrie cDNA Resource Center (Missouri University of Science and Technology, Rolla, MO). The peptide β ARKct, inhibits the activation of the G protein–coupled receptor kinase 2 which is required for G β γ functionality. VSMC were plated at 70% confluence in 100 mm dishes and allowed to grow overnight. Recombinant adenovirus was then added at the appropriate concentrations (β ARK_{CT}; 1000 MOI) in a reduced volume of media (1.5 – 2 mL). After 48 hour incubation, the media was changed and cells grown for an additional 24 hours. The cells were then used for experimentation. Empty vector served as control.

siRNA Transfection

Pre-designed HPLC-purified siRNA for gene knockdown for the N-formyl-Met-Leu-Phe receptors *FPR* and *FPRL1* were procured commercially. VSMC of 50% confluence in 60 mm plates are starved overnight in 4 mL of Opti-MEM reduced serum medium (Life Technologies, Inc., Carlsbad, CA). siRNA was transfected using Lipofectamine 2000

(Invitrogen, Inc., Carlsbad, CA) following product protocol. Briefly, 22 μ L of Lipofectamine 2000 was first incubated in total volume of 250 μ L of Opti-MEM for 5 minutes at room temperature. It was then added to 250 μ L of Opti-MEM containing 440 pmoles of siRNA. The solution was mixed gently and incubated for 20 minutes at room temperature, after which it was added to the starved plates. The medium was changed after 4–6 hours of incubation. The cells were used between 24–72 hours after transfection for cell assays. Scrambled siRNA served as a control. Using the methodologies described, we conducted concentration-dependent experiments with siRNA against either *FPR* or *FPRL1* and demonstrated a concentration-dependent decrease in protein expression that was specific for the protein targeted without altering the expression of the other proteins.

Gelatin and Reverse Gelatin Zymography

Gelatin zymography for matrix metalloproteinases (MMPs) was performed as described previously (13). Reverse Gelatin zymography was performed as described previously (14) with minor modifications. Bands representing the intact zymogens (as defined by the standards) were quantified by means of scanning of gels with an HP3C Deskscan (Hewlett-Packard, Palo Alto, CA) and analyzed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (s.e.m.) and statistical differences between groups were analyzed using one-way ANOVA with post hoc Dunnett's multiple comparisons correction where appropriate. A P-value of < 0.05 was considered significant.

RESULTS

Cell migration

To examine the migration responses of VSMC to suPAR, both linear wound assays and Boyden chamber models were used. suPAR induced migration of VSMC in both the wound and Boyden chamber assays which was weaker than that stimulated by ATF (Fig 1A and 1B). To determine if the suPAR responses were G-protein dependent, VSMC were incubated with the G α i inhibitor, PTx (100ng/ml), or transfected with the G $\beta\gamma$ inhibitor, β ARK_{CT}. suPAR induced-migration of VSMC was blocked by both G-protein inhibitors, suggesting that the responses are G α i and G $\beta\gamma$ dependent (Fig 1A and 1B)). The migration responses of VSMC to ATF were similarly dependent on these G-proteins (Fig 1A and 1B)). Removal of the full uPAR molecule from the surface of the VSMC by incubation of the cells with a PI-phospholipase C (15) did not interfere with the response to suPAR but blocked the response to ATF (Fig 1A and 1B)). suPAR-induced migration of VSMC was independent of plasmin activity (i.e. preincubation with plasmin inhibitors, EACA and aprotinin had no effect) but was dependent on MMP activity (i.e. inhibited by GM6001; Fig 1C and 1D). The responses to ATF were equivalent to suPAR responses under these conditions (Fig 1C and 1D).

MAPK and EGFR activation

To examine the role of the MAPKs, ERK1/2, p38^{MAPK} and JNK, VSMC were incubated with the chemical inhibitors PD98059 (ERK inhibitor), SB203580 (p38^{MAPK} inhibitor) and SP600125 (JNK inhibitor). suPAR-mediated cell migration was inhibited by these respective inhibitors for the MAPKs (ERK1/2, p38^{MAPK} and JNK) (Fig 1E and 1F). The responses to ATF were similar to those found for suPAR (Fig 1E and 1F). To examine the activation of MAPKs, multiplex assays for the respective active and total kinases was performed. suPAR induced activation of ERK1/2 (Fig 2A), p38^{MAPK} (Fig 2B) and JNK (Fig 2C) in the VSMC. This activation was G α i and G β γ dependent, in that it was inhibited by pretreatment with the G α i inhibitor, PTx, and with transfection of the G β γ inhibitor, β ARK_{CT} (Fig 2A, 2B and 2C). suPAR activation of the MAPKs was, however, independent of removal of the uPAR molecule from the surface of the cells by incubation of the cells with a phospholipase. suPAR activation of the MAPKs was independent of plasmin activity (i.e. EACA and aprotinin had no effect) and MMP activity (i.e. GM6001 had no effect). suPAR did not activate EGFR as determined by western blotting.

fMLP receptors and suPAR

suPAR has been shown to mediate its responses through formic (fMLP) receptors in non vascular cells. To examine migration in response to fMLP, we incubated fMLP with VSMC in the wound assay and the Boyden chamber (Fig 1A to 1D). fMLP induced migration of the VSMC in both assays. Following incubation with G α i G-protein inhibitor, PTx or transfections with the G β γ inhibitor, β ARK_{CT}, migration to fMLP was inhibited by both agents, suggesting that G-protein G α i and G β γ inhibitors are required for migration in response to fMLP. fMLP-mediated migration was unaffected by removal of uPAR suggesting that uPAR has no role in its migration signaling pathway. Interruption of the low affinity formic receptor *FPRL1* with siRNA to *FPRL1* reduced cell migration in response to fMLP and prevented cell migration in response to suPAR (Fig 3A), while interruption of high affinity formic receptor *FPR* with siRNA to *FPR* prevented cell migration in response to fMLP but had no effect on VSMC migration mediated by suPAR (Fig 3B). Scrambled siRNA had no effect. MAPK (ERK1/2, p38^{MAPK} and JNK) activation occurred in response to fMLP and these responses were strongly inhibited by siRNA to *FPR* and weakly inhibited by siRNA to *FPRL-1* (Fig 3C, 3D and 3E). Following incubation with the G α i inhibitor, PTx or transfections with the G β γ inhibitor, β ARK_{CT}, fMLP-induced activation of MAPKs (ERK1/2, p38^{MAPK} and JNK) was inhibited by both agents suggesting that the responses were G α i/G β γ dependent (Fig 2A, 2B and 2C). fMLP induced activation of ERK1/2, p38^{MAPK} and JNK in a similar manner to suPAR (Fig 3C, 3D and 3E). MAPK activation in response to suPAR was strongly inhibited by siRNA to *FPRL-1*, while siRNA to *FPR* had no effect (Fig 3C, 3D and 3E).

Proteases and suPAR

Application of suPAR to the VSMC induced the activation of MMP-2 and increased the expression of MMP-2 after 24 hours (Fig 4A and B). There was no change in MMP-9, TIMP-1 or TIMP-2 expression or activity (data not shown). Inhibition of ERK1/2 with PD98059 (ERK inhibitor) reduced MMP-2 activation and expression by suPAR and fMLP

(Fig 4A and 4B). Incubation with the p38^{MAPK} inhibitor, SB203580, and JNK inhibitor, SP600125, did not significantly effect MMP-2 activation and expression by suPAR (Fig 4A and 4B). Incubation with the p38^{MAPK} inhibitor, SB203580, but not JNK inhibitor, SP600125, significantly reduced MMP-2 activation and expression by fMLP (Fig 4A and 4B). Interruption of the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL-1*) reduced MMP-2 activation and expression in response to suPAR but not to fMLP (Fig 4C and 4D) while interruption of high affinity N-formyl-Met-Leu-Phe receptor (FPR) with siRNA prevented MMP-2 activation and expression in response to fMLP but not to suPAR (Fig 4C and 4D).

DISCUSSION

This study demonstrates that the suPAR fragment induces migration of VSMC through the low affinity *FPRL-1* receptor, using a G-protein MAPK kinase-mediated process that involves the expression and release of the gelatinase MMP-2 but does not require EGFR. Activation of ERK1/2 was necessary for MMP-2 expression and release while activation of p38^{MAPK} and JNK is thought to effect the cytoskeletal interactions required for cell migration. We have shown that ATF will induce plasmin-independent cell migration and that this migration is ERK1/2 and p38^{MAPK} dependent (3). We have previously demonstrated the intact uPA molecule induces time-dependent phosphorylation of EGFR, which was dependent on plasmin activity (6). Inhibition of EGFR reduced both ERK1/2 and p38^{MAPK} activation. We have also demonstrated that uPA activation of PI3K and MKK3/6 was *erbB1*-dependent but that of MEK1 was *erbB1*-independent (6). Migration in response to uPA is dependent on the aminoterminal domain of the molecule and we have demonstrated that ATF, the fragment of uPA that carries the aminoterminal domain, will induce VSMC migration through EGFR (3). This response is mediated by Gβγ G-proteins to activate ADAM-9 and -10 (7).

Urokinase requires binding of its aminoterminal domain to uPAR on the cell surface to initiate migration. If one uses ATF instead, ATF induces plasmin-independent cell migration in VSMC (3). This migration requires the binding to the GPI-tethered uPAR receptor. uPAR exists on the membrane in a complex of multiple receptors including LRP, integrins and *erbB1*. Our recent data has demonstrated that ATF will induce smooth muscle cell migration through transactivation of *erbB1* in an ADAM-dependent manner and is MAPK dependent (3, 6, 7). It appears that suPAR does not trigger the EGFR-mediated pathway but is required for the MAPK responses that lead to cell migration. ATF binds uPAR, which induces cleavage of the uPAR receptor into a cell-bound D1 fragment and a soluble D2/D3 fragment, soluble uPAR (suPAR). D2/D3 then binds to the low affinity receptor of fMLP, *FPRL1*, to induce a Gαi-mediated response (8). The current study demonstrates that suPAR induces cell migration in human VSMC and that this migration is mediated by MAPK (ERK1/2, p38^{MAPK} and JNK), but in distinction to ATF, it is not mediated by an EGFR pathway. Two forms of uPAR are present on the cell surface: full-length and cleaved uPAR, each specifically interacting with one or more transmembrane proteins. In a recent study (16), both wild-type (wt) and cleavage resistant-uPAR were shown to be able to mediate uPA-induced migration, and were constitutively associated with the EGFR, and associated with α3β1 integrin upon uPA binding. However, they engage different pathways in response

to uPA. wt-uPAR requires both integrins and FPRL1 to mediate uPA-induced migration, and association of wt-uPAR to $\alpha 3\beta 1$ results in uPAR cleavage and extracellular signal-regulated kinase (ERK1/2) activation. On the contrary, cleavage resistant-uPAR does not activate ERK1/2 and does not engage FPRL1 or any other G protein-coupled receptor, but it activates an alternative pathway initiated by the formation of a triple complex (uPAR- $\alpha 3\beta 1$ -EGFR) and resulting in the autotyrosine phosphorylation of EGFR (16).

This study has demonstrated that fMLP induced activation of ERK1/2, p38^{MAPK} and JNK in the VSMC through G α i and G $\beta\gamma$ -mediated pathways. fMLP has been shown to bind to two different G protein-coupled receptors (FPR) and the low affinity FPRL1 to initiate a signal transduction cascade leading to cell activation and migration. In fibroblasts, fMLP requires G α i coupled, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) activation to induce intracellular calcium flux and a transient increase in F-actin in order to facilitate cell migration (17, 18). fMLP also can induce MAPK activation through G α i2 G-proteins in these cells (19, 20). fMLP-stimulated ERK1/2 activity is dependent on tyrosine kinases, PI3-K, PKC, and phospholipase C; p38^{MAPK} activation was dependent on PI3-K and phospholipase C; while JNK activation was independent of all of these signaling components. Both ERK1/2 and p38^{MAPK} are required for the generation of reactive oxygen species (20). Similar to uPA, soluble and membrane-bound fibronectin greatly increased fMLP-induced chemotaxis (21, 22). In granulocytes, fMLP induces migration through cell polarization, the generation of reactive oxygen species, the production of arachidonic acid metabolites and the release of lysosomal enzymes (23). Both fMLP receptors are present on coronary VSMC and fMLP can modulate human coronary arterial tone through the generation of Thromboxane A₂ and Prostaglandin I₂ (24).

A major role of MMPs is to enable vascular remodeling through decomposition of the existing ECM scaffold, while a new ECM is synthesized and organized. MMP-2 is constitutively expressed in VSMC and has been shown to be required for SMC migration (25, 26). It has been demonstrated that there is a rapid upregulation of MMP-2 production after mechanical injury in the rat injury model (27, 28). We have demonstrated that MMP-2, a key protease in cell migration, is also induced by suPAR and fMLP. MMP-2 is closely associated with cell migration and is a target of uPA. In contrast to MMP-2, MMP-9 is not constitutively expressed in VSMC but is induced after arterial injury and this increase in activity coincides with the onset of cell proliferation (27, 29–31). suPAR did not stimulate MMP-9 expression. MMP inhibitors decrease smooth muscle cell proliferation and migration (27, 32, 33). The regulation of TIMPs is distinctly different from MMPs. TIMP-2 is more closely associated with MMP-2 and TIMP-2 is constitutively expressed in VSMC and the vessel wall. suPAR did not change TIMP-2 expression. suPAR, through the low affinity fMLP receptor, induced MMP-2 expression and activation. suPAR utilized ERK1/2 to induce MMP-2 expression. In contrast, fMLP utilized its high affinity receptor to induce MMP-2 expression through a pathway mediated by activated ERK1/2 and p38^{MAPK}.

Conclusion

suPAR induces human smooth muscle cell activation and migration independent of the full uPAR through activation of the G protein-coupled receptor **FPRL1** which is not linked to

the plasminogen activation cascade. This results would suggest that identifying and targeting the down stream receptors (i.e. low affinity fMLP receptor) in the uPAR pathway can selectively inhibit VSMC migration.

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A

B

C

D

E

F

Figure 1. Cell migration

suPAR induced migration of VSMC in the wound and Boyden chamber assays (**A and B**). Incubation with the G α i inhibitor, pertussis toxin (PTx, 100ng/ml), or G $\beta\gamma$ inhibitor, β ARK_{CT} (MOI 50) blocked the response to suPAR (10nM) in both assays (**A and B**). The empty virus (EV) had no effect. The responses to ATF (10nM) in the wound and Boyden chamber assays were similar to those seen for suPAR (**A and B**). Removal of the full uPAR molecule from the surface of the cells by incubation of the cells with a Phosphatidylinositol-specific-phospholipase C (100 μ g/mL) did not interfere with the response to suPAR but blocked the response to ATF (**A and B**). Incubation of VSMC with the plasmin inhibitors ϵ -aminocaproic acid (EACA, 100 μ M) and aprotinin (100units/ml) had no effect on suPAR-induced migration (**C and D**). Incubation of VSMC with GM6001 (10nM), an MMP inhibitor blocked suPAR-induced migration of VSMC in the wound and Boyden chamber assays (**C and D**). The responses to ATF were equivalent to suPAR responses under these conditions (**C and D**). fMLP (10nM) induced migration of the VSMC (**A and B**), which was inhibited by G-protein G α i and G $\beta\gamma$ inhibitors (**A and B**) and unaffected by removal of uPAR (**A and B**). Incubation of VSMC with GM6001, an MMP inhibitor, blocked fMLP-induced migration of VSMC while incubation with the plasmin inhibitors EACA and aprotinin had no effect (**C and D**). The respective inhibitors for ERK1/2 (PD98059, PD,

25 μ M), p38^{MAPK} (SB203580, SB, 10 μ M) and JNK (SP600125, SP, 1 μ M) inhibited suPAR-mediated cell migration (**E and F**). Similar responses to the MAPK inhibitors were seen with ATF and fMLP. All values are the mean $\pm$ s.e.m. percent of the control for five experiments (* p<0.05, **p<0.01 compared to respective control agonist; § p<0.01 compared to suPAR).

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Figure 2. MAPK activation

suPAR (10nM) induced ERK1/2 (A), p38^{MAPK} (B) and JNK (C) activation, which was G α i (PTx sensitive, 100ng/ml) and G β γ (β ARK_{CT} sensitive) dependent. fMLP (25 μ M) also induced activation of ERK1/2(A), p38^{MAPK} (B) and JNK (C) activation in a similar manner, which was G α i/G β γ dependent. The empty virus (EV) had no effect. Data represents the peak activity of the MAPKs at the same time as determined from time course experiments using multiplex assays. All values are the mean $\pm$ s.e.m. percent of the control for five experiments using the multiplex assay system (* p<0.05, **p<0.01 compared to respective control agonist).

A

B

C

suPAR	+	+	+	+		-	-	-	-
fMLP	-	-	-	-		+	+	+	+
siRNA to FPR	-	+	-	-		-	+	-	-
siRNA to FPRL-1	-	-	+	-		-	-	+	-
scrRNA	-	-	-	+		-	-	-	+

D

suPAR	+	+	+	+		-	-	-	-
fMLP	-	-	-	-		+	+	+	+
siRNA to FPR	-	+	-	-		-	+	-	-
siRNA to FPRL-1	-	-	+	-		-	-	+	-
scrRNA	-	-	-	+		-	-	-	+

E

Figure 3. fMLP receptors and suPAR

Interruption of the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL1*) reduced cell migration in response to fMLP (25 μ M) and decreased cell migration in response to suPAR (10nM) (A) while interruption of high affinity N-formyl-Met-Leu-Phe receptor (FPR) with siRNA prevented cell migration in response to fMLP but not to suPAR (B). MAPK activation in response to fMLP was strongly inhibited by siRNA to FPR and weakly inhibited by siRNA to FPRL-1 using the multiplex assay system (C, D and E). MAPK activation in response to suPAR was strongly inhibited by siRNA to FPRL-1, while siRNA to FPR had no effect using the multiplex assay system (C, D and E). fMLP induced activation of ERK1/2, p38^{MAPK} and JNK in a similar manner to suPAR and this activation was also G α i/G β γ dependent (C, D and E). All values are the mean $\pm$ s.e.m. percent of the control for five experiments (* p<0.05, **p<0.01 compared to respective control agonist). scrRNA, scrambled siRNA.

A

MMP-2 Activity

Fold increase in
Ratio Active /Total Protein

suPAR

fMLP

B

suPAR	+	+	+	+		-	-	-	-
fMLP	-	-	-	-		+	+	+	+
PD98059	-	+	-	-		-	+	-	-
SB203580	-	-	+	-		-	-	+	-
SP600125	-	-	-	+		-	-	-	+

C

suPAR	+	+	+	+		-	-	-	-
fMLP	-	-	-	-		+	+	+	+
siRNA to FPR	-	+	-	-		-	+	-	-
siRNA to FPRL-1	-	-	+	-		-	-	+	-
scrRNA	-	-	-	+		-	-	-	+

Figure 4. Proteases and suPAR

Application of suPAR (10nM) to the VSMC cells induced the proteolytic activity of MMP-2 as determined by zymography (**A**) and increased the protein expression of MMP2 after 24 hours as determined by multiplex assay (**B**). Inhibition of ERK1/2 with PD98059 (ERK inhibitor, PD, 25μM) reduced both MMP-2 proteolytic activation and protein expression (**A and B**). Incubation with p38^{MAPK} and JNK with SB203580 (p38^{MAPK} inhibitor, SB, 10μM) and SP600125 (JNK inhibitor, SP, 1μM) respectively did not significantly effect MMP-2 and MMP-9 proteolytic activation and protein expression (**A and B**). Interruption of the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL1*) with siRNA reduced MMP-2 proteolytic activation and protein expression in response to fMLP (25μM) (**C and D**) while interruption of high affinity N-formyl-Met-Leu-Phe receptor (FPR) with siRNA prevented MMP-2 proteolytic activation and protein expression in response to fMLP but not to suPAR (**C and D**). Scrambled siRNA (scrRNA) had no effect. All values are the mean ± s.e.m. percent of the

control for five experiments using the multiplex assay system (* $p < 0.05$, ** $p < 0.01$ compared to respective control agonist).

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