JOURNAL OF SURGICAL RESEARCH 195 (2015) 396–405

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Association for Academic Surgery

Role of formic receptors in soluble urokinase receptor–induced human vascular smooth muscle migration

*Enrico A. Duru, PhD, Yuyang Fu, MD, PhD, and Mark G. Davies, MD, PhD, MBA**

Vascular Biology and Therapeutics Program, Houston Methodist Research Institute, Houston, Texas

ARTICLE INFO

Article history:
Received 19 August 2014
Received in revised form
31 December 2014
Accepted 4 February 2015
Available online 12 February 2015

Keywords:
uPA
Soluble uPAR
Migration
Cell signaling
Human coronary smooth muscle cell

ABSTRACT

Background: Vascular smooth muscle cell (VSMC) migration in response to urokinase is dependent on binding of the urokinase molecule to the urokinase plasminogen receptor (uPAR) and cleavage of the receptor. The aim of this study was to examine the role of the soluble uPAR (suPAR) in VSMC 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 used to study these pathways. Assays were performed for mitogen-activated protein kinase and epidermal growth factor receptor activation.

Results: suPAR induced concentration-dependent migration of VSMC, which was G protein-dependent and was blocked by Gαi and Gβγ 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 c-Jun N-terminal kinase [JNK] activation in a Gαi/Gβγ-dependent manner, and interruption of these signaling pathways prevented suPAR-mediated migration. suPAR activity was independent of plasmin activity. suPAR did not activate epidermal growth factor receptor. 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 matrix metalloproteinase-2 expression and activity, and this was dependent on the low affinity N-formyl-Met-Leu-Phe receptor (*FPRL1*) and ERK1/2.

Conclusions: 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.

Presented in part at fourth Academic Surgical Congress (Sanibel Harbor, Florida, February, 2009) and sixth Academic Surgical Congress, Huntington Beach, California (February, 2011).

* *Corresponding author*. Houston Methodist Research Institute, 6670 Bertner Ave, Houston, TX 77030. Tel.: (713) 441-1261; fax: (713) 790-3755.

E-mail address: mdavies@tmhs.org (M.G. Davies).

0022-4804/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2015.02.003

1. 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 microenvironment of the cell wall, uPA can also be cleaved by other proteases into several biologically active fragments as follows: aminoterminal fragment (ATF), kringle domain (K), and carboxy-terminal fragment. Each appears to have unique characteristics [3–5]. We have shown that ATF will induce plasmin-independent cell migration and that carboxy-terminal fragment 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, integrins, and epidermal growth factor receptor (EGFR). Our recent data have demonstrated that ATF will induce smooth muscle cell (VSMC) migration through transactivation of *erb*B1 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 Gαi-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 was to test the hypothesis that *FPRL1* is required for suPAR-induced cell signaling and suPAR-mediated cell migration in human VSMC.

2. Methods

2.1. 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 were used. Assays were performed for MAPK and EGFR activation. Chemical and molecular inhibitors to G-protein signaling, EGFR, fMLP receptors, and MAPK activation were used to study these pathways.

2.2. Materials

suPAR and ATF were purchased from American Diagnostica, Inc (Greenwich, CT). fMLP, pertussis toxin (PTx) (Gαi inhibitor, 100 ng/mL), phosphatidylinositol-specific-phospholipase C (100 μg/mL), PD98059 (ERK inhibitor, 25 μM) SB203580 ($p38^{MAPK}$ inhibitor, 10 μM), SP600125 (c-Jun N-terminal kinase [JNK] inhibitor, 1 μM), EGFR inhibitor (AG1478, 10 μM), ε-aminocaproic acid (EACA; plasmin inhibitor, 100 μM), aprotinin (plasmin inhibitor, 100 units/ml), and GM6001 (matrix metalloproteinase [MMP] inhibitor, 10 nM) 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-extracellular signal-regulated kinase (ERK1/2) antibody was purchased from Promega, Inc (Madison, WI). Total ERK1/2 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 (Beverley, MA). Dulbecco minimal essential medium (DMEM) and Dulbecco phosphate-buffered saline were purchased from Corning cellgro (Corning, NY).

2.3. Wound assay

The wound assay was performed with VSMC as previously described [3,6]. Human VSMC were grown to confluence in 60 mm^2 dishes and then starved (1% serum) for 24 h in the presence of hydroxyurea (5 mM, Sigma Chemical Co) to prevent proliferation. Thereafter, each dish was divided into a 2 × 3 grid. With the use of a 1–200 μL pipette tip, a linear wound was made in each hemisphere of the dish. Immediately after wounding, media were changed to fresh DMEM (for all reagent dishes and as negative control) or 10% fetal bovine serum (positive control). Cells were then allowed to migrate over 24 h at 37°C in DMEM with or without suPAR (10 nM), ATF (10 nM), or fMLP (10 nM). 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 lens with an attached SPOT camera (Diagnostic Instruments, Inc, Sterling Heights, MI), 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 h at 37°C. Each field was measured at time 0 and at 24 h. The area of each field was measured using SPOT Advanced software (Diagnostic Instruments, Inc), 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.

2.4. Boyden chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuro Probe, Inc, Gaithersburg, MD) and polycarbonate filters (Neuro Probe, Inc, 10 μm pore size, 25 × 80 mm, polyvinylpyrrolidone free) with VSMC as previously described [3,6]. suPAR (10 nM), ATF (10 nM), or fMLP (10 nM) was added to