Urokinase Receptor-Dependent Upregulation of Smooth Muscle Cell Adhesion to Vitronectin by Urokinase

Alan W. Chang, Alice Kuo, Elliot S. Barnathan, S. Steve Okada

Abstract—The plasminogen activator system has been implicated in the modulation of the response to vascular injury. Although urokinase-type plasminogen activator (uPA) and its receptor (uPAR) may enhance matrix degradation as well as migration and invasion by smooth muscle cells (SMCs), their roles in cell adhesion are uncertain. Therefore, we examined the ability of uPA and uPAR to modulate adhesion of cultured human vascular SMCs to various matrices. We demonstrated a dose-dependent stimulation of adhesion by single-chain uPA (scuPA) to vitronectin (maximum 1.55-fold [±0.04-fold] increase, 10 nmol/L, \(P<0.002\)) but not to laminin, collagen I, or collagen IV. Baseline adhesion to vitronectin was completely inhibited by both EDTA and RGD peptide but was restored to >40% of control in the presence of scuPA (\(P=0.001\) and 0.046, respectively). Adhesion to vitronectin was also significantly enhanced by the amino-terminal fragment of uPA (\(P=0.007\)) and two-chain, high-molecular-weight uPA (\(P<0.01\)) but not by the low-molecular-weight fragment of uPA, which lacks the receptor-binding domain. Aprotinin, a plasmin inhibitor, had no effect on baseline or scuPA-stimulated adhesion, suggesting a plasmin-independent process. Preincubation of scuPA with soluble uPAR inhibited scuPA stimulation of adhesion by 88±14% (\(P=0.01\)), as did pretreatment of SMCs with phosphatidylinositol-specific phospholipase C, which removes glycosphatidylinositol-anchored proteins, including uPAR. Antibodies to both \(\alpha_\beta_3\) and \(\alpha_\beta_4\) integrin inhibited baseline adhesion but not scuPA stimulation. Finally, coating plates with scuPA alone enabled cell adhesion, which could be inhibited by both soluble uPAR and anti-uPAR antibodies. These data suggest that uPA stimulates adhesion of SMCs specifically to vitronectin and that it is mediated by an interaction with uPAR. Upregulation of both proteins after vascular injury may facilitate migration through stimulation of both matrix degradation and cell adhesion. (Arterioscler Thromb Vasc Biol. 1998;18:1855-1860.)

Key Words: smooth muscle cell adhesion • upregulation • urokinase • vitronectin • urokinase receptor

After vascular injury, smooth muscle cells (SMCs) proliferate within the media of the vessel and migrate to the intima, where they continue to proliferate and contribute to atheroma formation and restenosis, common problems after vascular procedures.1 Adhesive functions of vascular SMCs play a central role in this inflammatory process. Cells use receptors expressed on the cell surface, growth factors produced by cells, and matrices secreted into the extracellular milieu.2–5

Traditionally, integrins have been thought of as the major cellular receptors mediating adhesion.6 SMCs bind to fibronectin, laminin, collagen I, and collagen IV primarily by means of \(\beta_1\) integrins.7–9 After vessel injury, however, SMCs use a different set of integrins and matrices for adhesion and migration.10 Vitronectin, a serum protein that inhibits complement activation and binds heparin, localizes to atherosclerotic plaques.11 Vitronectin has been shown to mediate migration of cells in vitro, suggesting that cells may use vitronectin to adhere and migrate after vascular injury. The major integrin receptors for vitronectin are \(\alpha_\beta_3\), \(\alpha_\beta_5\), \(\alpha_\beta_6\), and \(\alpha_\beta_7\). In carcinoma cells, \(\alpha_\beta_5\) promotes cell attachment to vitronectin but not migration. When transfected with \(\beta_n\), these cells are capable of migrating on vitronectin,12,13 demonstrating that cells use different cellular receptors in response to various stimuli. Glioblastoma cells are thought to use vitronectin and \(\alpha_\beta_3\) receptor to invade normal brain tissue.14 Abciximab, a monoclonal antibody that binds with equal affinity to both \(\alpha_\beta_3\) and \(\alpha_\beta_4\) receptor, is used in humans to reduce ischemic complications and possibly to reduce restenosis after angioplasty.15 RGD peptide, which blocks ligand binding of various integrin receptors (including \(\alpha_\beta_3\) and \(\alpha_\beta_6\) receptors), inhibited neointima formation in damaged hamster carotid arteries, presumably by interfering with these receptors on platelets and SMCs.16 Thus, SMCs appear to respond to vascular injury by using \(\alpha_\beta_3\) and \(\alpha_\beta_4\) integrin receptors to adhere to and migrate on vitronectin, which localizes to areas of vessel damage.13 Upregulation of these factors by SMCs represents a potential target for therapies to limit the vascular injury response. Recently, the plasminogen activator system has been suggested to play a role in atheroma formation and regulation of cellular adhesion. Tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and uPA recep-
tor (uPAR) are upregulated in atherosclerotic lesions and are localized to macrophages and neointimal SMCs. UPA and uPA are serine proteases secreted by migrating cells. They convert plasminogen to plasmin, which mediates cellular migration and matrix degradation, and aid in a diverse number of cellular processes, such as angiogenesis and tumor invasion. uPAR has also been implicated as a nonintegrin cellular adhesion receptor. uPAR, which is expressed on a wide variety of cells (including human SMCs), is a glycoprotein with a molecular mass of \( \approx \) 45 to 60 kDa and a glycoposphatidylinositol (GP) anchor. Recombinant uPAR binds vitronectin, and this binding is augmented by the addition of uPA. Transfected epithelial cells expressing membrane-anchored uPAR become significantly more adhesive than cells expressing soluble receptor. Cytokine-stimulated myelomonocytic cells adhere to vitronectin in an RGD-independent manner, and this adhesion is tightly linked to uPAR occupancy.

We sought to define the factors, related to both integrin and plasminogen activator, involved in SMC adhesion to various matrices given the central role of SMCs in atheroma formation and recent evidence linking the plasminogen activator system to cellular adhesion. We demonstrate that uPA stimulates SMC adhesion selectively to vitronectin in a plasmin-independent but uPAR-dependent manner. We also demonstrate that uPA can act as an adhesive substrate for SMCs.

### Methods

**Reagents**

Human collagen I, laminin, collagen IV, and anti-human integrin \( \alpha_\text{b} \beta_3 \) antibody P1F6 (unpurified, from mouse ascites fluid) were obtained from Gibco BRL. Murine chimeric antibodies against human integrin \( \alpha_\text{b} \beta_3 \) and \( \alpha_\text{v} \beta_3 \) (abiximab and 7E3, respectively) and anti-human \( \alpha_\text{v} \beta_3 \) antibody (10E5) were supplied by Centocor (gift of M. Nakada). Human vitronectin was obtained from Promega. Murine monoclonal antibody 3936 against human uPAR was obtained from American Diagnostica (gift of J. Bognacki). Single-chain uPA (scuPA), two-chain, high-molecular-weight uPA (tcuPA), the low-molecular-weight (LMW) fragment of uPA, the amino-terminal fragment of uPA (ATF), and recombinant soluble uPAR (suPAR) were obtained from Abbott Laboratories (gifts of A. Mazar and J. Henkin). Higazi et al.28 showed that the suPAR preparation does not cleave scuPA. Phosphatidylglycerol-specific phospholipase C (PIPLC) was provided by M. Low (Columbia University). RGD peptide was purchased from Sigma. The sequence of the RGD peptide was Arg-Gly-Asp-Ser.

**Cells**

SMCs were grown using the explant technique from human umbilical veins or human adult aorta as previously described. All use of human tissue was approved by our institutional review board. In brief, an umbilical vein or aortic media was isolated surgically and minced on culture plates. Unless otherwise noted, experiments were performed with human umbilical vein SMCs (HUV/SMCs). SMCs were grown in a mixture of Dulbecco’s modified Eagle’s medium (DMEM) and F12 Ham’s solution supplemented with 10% FBS, penicillin, streptomycin, and Fungizone. Cells were seeded at confluence with trypsin-EDTA. Passages 3 to 12 were used for experiments.

**Assay for Cell Adhesion**

Nunc microwell 96-well plates were coated with matrix for 1 hour at 37°C and then blocked with 1% BSA-PBS for 30 minutes at 37°C. SMCs were removed with 2 mmol/L EDTA in calcium- and magnesium-free PBS after they were washed with calcium- and magnesium-free PBS. Cells were resuspended in Hank’s balanced salt solution with 0.1% BSA. Unless otherwise specified, reagents and SMCs (100 μL/well, at a concentration of 2×10⁵ cells/mL) were added directly to wells and allowed to incubate for 2 hours at 37°C. Cells were then washed with PBS 3 times and fixed with 10% formaldehyde and 0.1 M acetate buffer (pH 5.2) for 15 minutes at room temperature. Cells were washed twice with acetate buffer, twice with deionized water, and allowed to air-dry. Fixed cells were stained with 0.05% naphthol blue-black acetate buffer for 30 minutes at room temperature. Cells were washed 3 times with deionized water and allowed to air-dry. Stain was then solubilized with 0.1 N NaOH and shaken for 30 minutes at room temperature. Absorbance was measured at 630 nm. All experiments were performed in triplicate with data representing average optical density minus background. Student’s t test was used to compare treatment groups with control cells with \( P < 0.05 \) considered significant.

### Results

**Effect of scuPA on HUVSMC Adhesion**

Given the recent evidence suggesting a role of the plasminogen activator system in cellular adhesion, we investigated the effect of scuPA on SMC adhesion to different matrices. Plates were coated with 5 μg/mL (67 nmol/L) vitronectin, 5 μg/mL (40 nmol/L) collagen I, 5 μg/mL (5 nmol/L) laminin, or 5 μg/mL (28 nmol/L) collagen IV. Simultaneous addition of 10 nmol/L scuPA with the cells to the well increased adhesion to vitronectin by 45±3% above the control level (\( P = 0.02 \)) (Figure 1). No significant increase in adhesion was seen with the other matrices, despite the various levels of absolute adhesion with different matrices. Stimulation of adhesion was dose-dependent (Figure 2, squares) and was seen with both HUVSMCs and adult aortic SMCs (not shown). At 10 nmol/L scuPA, adhesion was increased by 55±4% (\( P = 0.002 \)), with half-maximal stimulation between 0.1 and 0.2 nmol/L.

Integrin-mediated cellular adhesion to vitronectin is RGD-dependent and inhibited by EDTA. Baseline adhesion of SMCs to vitronectin was inhibited by 98±1.2% (\( P = 0.01 \)) and 98±0.4% (\( P = 0.01 \)) by 100 μmol/L RGD and 10 mmol/L EDTA, respectively. However, when scuPA was added to cells in addition to RGD or EDTA, adhesion was restored to 50±4% (\( P = 0.046 \)) and 56±2% (\( P = 0.001 \)) of the control level for RGD peptide and EDTA, respectively (Figure 3).
scuPA-mediated stimulation of SMC adhesion to vitronectin in the presence of RGD suggests that a nonintegrin cellular receptor may mediate this adhesion.

**Inhibition of SMC Adhesion With Anti-integrin Antibodies**

To better characterize the integrin receptors used by SMCs, we repeated the experiments in the presence of an antibody to αvβ5 (P1F6), αvβ3 (abciximab or 7E3), or αIIbβ3 (control, 10E5) (Figure 4). The level of inhibition varied from 13±5% (P=0.09) for abciximab to 70±2% (P=0.001) for P1F6. There was no inhibition of SMC adhesion to collagen I, laminin, or collagen IV induced by any of these antibodies (not shown). Thus, to adhere to vitronectin, SMCs use primarily αvβ5 integrin, although αvβ3 integrin also contributes to adhesion. Nevertheless, in the presence of P1F6, the level of adhesion increased in a dose-dependent manner from 51% to 110% of the control level (P=0.04) with the addition of scuPA (Figure 2, circles).

**SMC Adhesion to Vitronectin: Effect of Various Domains of uPA**

To further define the role of scuPA in augmenting SMC adhesion to vitronectin, ATF and LMW uPA were incubated with cells under the same conditions as scuPA. Equimolar concentrations of scuPA, ATF, LMW uPA, tcuPA, or a combination of ATF and LMW uPA were added to vitronectin-coated wells along with SMCs and incubated at 37°C for 2 hours. scuPA increased SMC adhesion by 41±5% above the control level (P=0.009), and active tcuPA increased adhesion by 35±6% (P=0.009). ATF, which possesses the receptor-binding domain of scuPA without catalytic activity, increased adhesion by 29±5% (P=0.007), whereas LMW uPA, which contains the catalytic site of scuPA but not the receptor-binding domain, had no effect on adhesion (data not shown). Finally, aprotinin, a plasmin inhibitor, was unable to significantly inhibit either baseline or scuPA-augmented adhesion (data not shown). These results suggest that the effect of scuPA on SMC adhesion to vitronectin is related to its binding to uPAR and does not require plasmin activity.
Inhibition of SMC Adhesion to Vitronectin by scuPA/suPAR Complex and PIPLC

On the basis of the data presented above, uPAR appears to function as a cellular adhesion molecule, mediating adhesion to vitronectin when scuPA is present. We sought to directly determine whether uPAR was necessary for the effect of scuPA on SMC adhesion by inhibition with suPAR, scuPA (10 nmol/L) was preincubated with increasing concentrations of suPAR (0, 0.5, 1, and 2 μmol/L) at 37°C for 1.5 hours. The scuPA/suPAR solution was then added to vitronectin-coated wells along with the SMCs and incubated for an additional 2 hours. Preincubation with suPAR significantly inhibited the stimulatory effect of scuPA (Figure 6) by 88±14% (P=0.01) at a 50-fold molar excess of suPAR. In the absence of scuPA, suPAR inhibited baseline adhesion by only 2±2% (P=0.42), indicating that suPAR inhibits only adhesion stimulated by scuPA. Prior studies by Higazi et al. demonstrated that the suPAR preparation does not cleave scuPA and thus excludes degradation of scuPA as a mechanism for inhibition by suPAR.

Similar results were obtained with PIPLC treatment of cells. PIPLC cleaves the GPI anchor of uPAR. Preincubation with 1 U/mL PIPLC for 1 hour at 37°C eliminated the increase in adhesion associated with scuPA (Figure 7). In PIPLC-treated SMCs in the presence of P1F6 antibody, scuPA stimulation was blunted but not eliminated.

SMC Adhesion to scuPA-coated Wells

Finally, to further investigate the ability of uPAR to act as a cellular receptor and to test whether scuPA could act as a ligand, wells were coated only with 20 μg/mL scuPA and blocked with 1% BSA-PBS. SMCs were then added to wells in the presence of various inhibitors. Cells were able to adhere and spread to scuPA-coated wells without difficulty and appeared normal. suPAR inhibited this adhesion by 95±1% (P=0.01). In addition, a monoclonal antibody to uPAR that blocks binding of uPA to uPAR (3936) inhibited adhesion by 21±3% (P=0.008). This adhesion to scuPA-coated wells was not inhibited by RGD peptide (5±3%, P=0.23). Control wells not precoated with scuPA did not support adhesion of SMCs at all (3±1% of control level) (Figure 8).

Discussion

These data demonstrate that uPA is capable of promoting SMC adhesion to vitronectin but not to other extracellular matrices. This stimulation is RGD- and EDTA-independent. In the presence of α5β1 and anti-α5β1 antibodies, scuPA was still able to increase SMC adhesion to vitronectin. Similarly, Waltz et al. using transforming growth factor-β1/D1-stimulated U937 cells, demonstrated a scuPA-mediated increase in adhesion to vitronectin independent of both EDTA and RGD peptide. Integrin-dependent adhesion to vitronectin is mediated by α5 receptors, primarily α5β1, α5β2, and α5β3. The α5 integrins recognize the RGD sequence in vitronectin, and the fact that RGD peptide does not inhibit the scuPA-mediated increase in SMC adhesion to vitronectin most likely points to a nonintegrin cellular receptor capable of promoting adhesion to vitronectin when properly stimulated by scuPA. Although upregulation of α5 integrin receptors may be an important component of the SMC response to scuPA stimulation.
vascular injury, the stimulatory effect of uPA on cellular adhesion appears to act independently of this system.

The effect of ATF and LMW uPA on SMC adhesion suggests that scuPA increases adhesion by means of receptor binding rather than protease activity. ATF, which contains the receptor-binding domain of scuPA, was able to significantly increase SMC adhesion to vitronectin, whereas the catalytic fragment, LMW uPA, had no significant effect on SMC adhesion. However, when added to ATF, LMW uPA seemed to inhibit the increase in adhesion seen with ATF alone. Low-affinity binding of LMW uPA to uPAR may cause conformational changes in uPAR and thus inhibit the binding of ATF to uPAR.

Stimulation of SMC adhesion to vitronectin by scuPA was inhibited by pretreatment of cells with PIPLC, suggesting that the cellular receptor involved is a GPI-anchored protein, such as uPAR. However, there was still a significant increase in SMC adhesion with scuPA after PIPLC treatment in the presence of P1F6. This may have been due to incomplete cleavage of uPAR from the cell surface, which was confirmed by binding experiments. When 125I-labeled scuPA binding to SMC was measured immediately after PIPLC treatment, there was an 80% reduction in binding but not a 100% reduction, suggesting that PIPLC treatment does not completely cleave all the receptors (S.S. Okada and E.S. Barnathan, unpublished data, 1991). In addition, SMCs may be able to regenerate uPAR after PIPLC treatment during the 2-hour incubation period. However, we cannot exclude the possibility of another scuPA-activated receptor that is not GPI-anchored mediating adhesion to vitronectin.

scuPA stimulation was also eliminated by preincubation of scuPA with suPAR, further implicating uPAR as a mediator of scuPA-induced adhesion. Whether preincubation with suPAR inhibits adhesion by directly blocking uPAR binding to sites on SMCs, by blocking vitronectin-binding sites, or by another mechanism remains to be determined. The fact that suPAR inhibited only adhesion stimulated by scuPA and not baseline adhesion suggests the first mechanism. Adhesion in the absence of scuPA may occur by means of a "lower-affinity" interaction with uPAR that is not significant enough to be inhibited by suPAR. In fact, studies have shown that suPAR binds to immobilized vitronectin in the absence of scuPA but that concurrent receptor binding of uPA markedly promotes binding to vitronectin.

The precise mechanism by which scuPA interacts with uPAR to mediate adhesion in general is not well understood. Wei et al. originally postulated that uPA stabilized an active conformation of uPAR. Kanse et al. demonstrated in endothelial cells that uPA increased vitronectin binding by increasing the affinity of vitronectin for uPAR. Moser et al., however, hypothesized that uPA mediated its effects by direct binding of uPA to vitronectin. Cytokines have been shown to augment the effect of uPA on cellular adhesion in monocytes, and monocyte adhesion is inhibited by cycloheximide and actinomycin D, which implies a receptor-mediated signal transduction pathway for the effect of uPA on cellular adhesion.

Recent studies have demonstrated that the uPAR may associate with various integrins. It has been suggested that the uPAR may interact directly with transmembrane inte-grins, which are in turn bound to the cytoskeleton, thus enabling interaction with the cytoskeleton. This interaction may occur independently of any RGD sites on the integrin receptors. Wei et al. found that uPAR complexes with β3 integrin and caveolin to inhibit native adhesion to fibronectin and to promote adhesion to vitronectin in human embryonic kidney cells transfected with uPAR cDNA. Other data have suggested a role of integrins in association with plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor of plasminogen activator activity. PAI-1 forms irreversible complexes with uPA and tPA and binds vitronectin. Stefansson et al. demonstrated that PAI-1 shares a vitronectin-binding site with αvβ3 integrin, suggesting that uPA increases cellular adhesion by binding to PAI-1, thereby exposing binding sites on vitronectin for αvβ3 integrin. Finally, data demonstrating that uPAR competes directly with PAI-1 for binding to vitronectin have been reported. Deng et al. mapped the uPAR-binding sequence in vitronectin to the somatomedin-binding domain, which contains the PAI-1-binding motif. Kjoller et al. recently demonstrated that PAI-1 inhibits cell migration by interfering with the binding of uPAR to vitronectin, independently of its function as a plasminogen activator inhibitor. Thus, uPA may increase adhesion by binding PAI-1 and freeing up binding sites within the vitronectin molecule for uPAR rather than for αvβ3.

Our data support a model of uPA stimulation of SMC adhesion (Figure 9) in which uPA binding to uPAR promotes adhesion to vitronectin by means of a conformational change in uPAR or exposure of a latent vitronectin-binding site on uPAR, the end result being increased affinity of uPAR for vitronectin. uPAR binding occurs at a site independent from the RGD sequence recognized by the αv β3 integrin receptors, explaining the upregulation of adhesion by urokinase in the presence of RGD peptide. Interaction between uPAR and integrin receptors, however, may still occur.

An unexpected finding was that scuPA supports adhesion of SMCs. This adhesion most likely uses uPAR as the adhesion receptor because the adhesion was inhibited by both suPAR and an anti-uPAR antibody but not an RGD peptide. The inhibition of adhesion by anti-uPAR antibody (3936) was only 21%, probably because 3936 is a monoclonal antibody that inhibits binding of uPA to suPAR but may not recognize
the site (or sites) on uPAR that mediates adhesion to the scuPA matrix. This result potentially defines a ligand role of urokinase with uPAR acting as its complementary cellular receptor. As such, uPA secreted by SMCs could promote adhesion and migration by acting as a matrix on which cells migrate, in addition to its well-known ability to activate plasminogen and generate plasmin, facilitating matrix breakdown. Interestingly, mice deficient in plasminogen, as well as those deficient in uPA, have reduced neointima formation after vascular injury. The novel role of scuPA-uPAR interaction could augment upregulation of cellular adhesion to vitronectin after vessel injury and contribute to the process of neointimal thickening. More importantly, it demonstrates the possibility that nonintegrin membrane receptor proteins may at times mediate cellular adhesion at discrete sites where ligand is present and facilitate cell-matrix interactions.

Acknowledgments
This work was supported by the National Institutes of Health (grants HL47839 to E.S.B. and HL02870 to S.S.O.). A.W.C. is a recipient of an American Heart Association Medical Student Research Fellowship. S.S.O. is a recipient of a National Institutes of Health Clinician Investigator Development Award.

References
Urokinase Receptor-Dependent Upregulation of Smooth Muscle Cell Adhesion to Vitronectin by Urokinase
Alan W. Chang, Alice Kuo, Elliot S. Barnathan and S. Steve Okada

*Arterioscler Thromb Vasc Biol.* 1998;18:1855-1860
doi: 10.1161/01.ATV.18.12.1855

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/18/12/1855

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/