Formation of Extracellular Matrix-Digesting Invadopodia by Primary Aortic Smooth Muscle Cells

Emilia Furmaniak-Kazmierczak, Scott W. Crawley, Rhonda L. Carter, Donald H. Maurice, Graham P. Côté

Abstract—Invasion of the subendothelial space by vascular smooth muscle cells (VSMCs) contributes to the development and progression of diverse cardiovascular diseases. In this report we show that the expression of activated versions of Src, Cdc42 and Rac1, or a kinase-dead but open form of the p21-activated kinase (PAK1), induces primary rat aorta VSMCs to form extracellular matrix-degrading actin-rich protrusions that are morphologically similar to the invadopodia formed by highly invasive tumor cells. The matrix-degrading structures are enriched in known markers for invadopodia, including cortactin and tyrosine-phosphorylated cortactin and contain the matrix metalloproteinases MMP-9 and MT1-MMP and the urokinase plasminogen activator receptor (uPAR). In contrast to other cell types, invadopodia formation in VSMCs is only weakly supported by the phorbol ester PBDu. Invadopodia formation by Src was dependent on Cdc42, Rac, and ERK, but not on p38 MAPK. Invadopodia formation induced by kinase-dead PAK1 required Src and ERK activity and a direct interaction with the exchange factor PIX. VSMCs embedded in a three-dimensional collagen matrix formed actin- and cortactin-rich extensions that penetrated through holes in the matrix, suggesting that invadopodia-like structures are formed in a three-dimensional environment. (Circ Res. 2007;100:1328-1336.)

Key Words: podosomes ■ invadopodia ■ Src ■ PAK ■ matrix metalloproteinase ■ vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) in the medial layer of healthy arteries are differentiated quiescent cells specialized for contraction. In response to vessel wall damage, contractile VSMCs can undergo a transition to a less-differentiated, synthetic phenotype that exhibits a greatly enhanced capacity to proliferate, migrate, and degrade the extracellular matrix (ECM). The ability to breach tissue barriers allows synthetic VSMCs to migrate to the subendothelial space, where they participate in the formation of neointimal lesions and contribute to the development and progression of cardiovascular diseases such as atherosclerosis and restenosis postangioplasty.

Highly invasive cell types often produce specialized actin-based structures termed podosomes or invadopodia that are involved in ECM adhesion and digestion. Although the exact relationship between these structures is uncertain, podosomes usually consist of a core column of actin filaments that extends upwards from the ventral cell surface into the cytoplasm whereas invadopodia are long filopodial-like membrane extensions that penetrate into the ECM. Podosomes and invadopodia are both associated with membrane invaginations that contain dynamin, suggesting a possible function as sites of endocytosis, exocytosis, or phagocytosis. Invadopodia enhance the invasive potential of cells by concentrating matrix metalloproteinases (MMPs) and serine proteases that degrade the ECM. It has been proposed that podosomes form when invasive cells are cultured on a solid surface, such as glass or plastic, that blocks cellular protrusions, whereas the more stable invadopodia develop when cells are cultured on an ECM. For the purposes of the current article, we will define podosomes as actin-rich columns that extend upwards into the cytoplasm and invadopodia as protrusive structures that penetrate into the ECM.

Treatment with the tumor-promoting phorbol ester phorbol 12,13-dibutyrate (PDBu) or expression of an activated form of the p21-activated kinase (PAK1) causes the A7r5 embryonic rat VSMC line to form podosomes. Although A7r5 cells express smooth muscle cell markers they differ in significant ways from primary VSMCs, including being nearly tetraploid. We have therefore tested whether primary rat aorta VSMCs are capable of forming podosomes or invadopodia. The results show that VSMCs are capable of forming invadopodia in response to activated versions of Src, Cdc42, Rac1, and PAK1, but not PDBu. Importantly, the invadopodia formed by VSMCs exhibit a much greater capacity to digest the ECM than do podosomes formed by A7r5 cells. Evidence is presented that VSMCs embedded within a three-dimensional (3-D) matrix of collagen type I extend large invadopodia-like structures.
Materials and Methods

Reagents and Plasmids

PDBu was from Sigma–Aldrich, SU6656 and bisindolylmaleimide I (BIM I) from Calbiochem and SB203580 and U0126 from Promega. Plasmids expressing Myc-tagged Cdc42V12, Cdc42N17, Rac1L61, and Rac1N17 were a gift of Dr A Hall (University College London, UK). Myc-tagged human PAK1 and the H83L/H86L/K299R mutant (PAK1-LL/R) were gifts from Dr W. T. Gerthoffer (University of Nevada).19 PAK1-LL and the PIX-binding mutant of PAK1-LL/R (P192G/R193A) were previously described.14 Expression plasmids for chicken Src-Y527F and Src-K295R,Y527F were a gift of Dr B. Elliot (Queen’s University, Canada).20

Cell Culture

VSMCs were isolated from the aortic arteries of adult rats by enzymatic digestion.21 Cells were grown on culture dishes or glass coverslips precoated with 20 μg/mL human fibronectin (Roche) in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 20% fetal bovine serum (FBS; Life Technologies Inc) and 1% penicillin-streptomycin (Hyclone). Experiments were performed using VSMCs between passages 2 and 5. A7r5 cells were grown in low glucose DMEM supplemented with 10% FBS and penicillin-streptomycin. VSMCs were transfected using Lipo-ffectamine Plus Reagent (Invitrogen) according to the manufacturer’s recommendations with efficiencies in the range of 10% to 20%. For 3-D culture, VSMCs (1 × 10⁴) were suspended in a solution composed of 0.6 mg/mL collagen I (BD Bioscience) in M199 and DMEM. Aliquots of 15 μL were spotted onto NITEX discs (type 03 to 100/44 fabric; Sefar).22 The collagen was allowed to gel at 37°C for 90 minutes. The discs were then turned over and covered with 15 μL of collagen solution without cells. After 90 minutes at 37°C the discs were placed in 96-well tissue culture dishes containing DMEM with 20% FBS.

ECM Degradation Assay

Glass coverslips were coated with cross-linked gelatin followed by 20 μg/mL fibronectin labeled using the FluoReporter FITC Protein Labeling kit (Molecular Probes).23 Before use coverslips were washed in 70% ethanol and incubated at 37°C in DMEM containing 20% FBS. VSMCs were cultured on the FITC-fibronectin-coated coverslips for 18 hours before transfection. The area of digestion was quantified from fluorescent images using Image ProPlus 5.0 software. Statistical analyses were performed using Microsoft Excel.

Immunofluorescence Microscopy

VSMCs were fixed in 2% paraformaldehyde in PBS, washed with PBS, and permeabilized with 0.2% Triton X-100. Cells were stained with TRITC-phalloidin (Sigma–Aldrich) and the following primary antibodies: rabbit anti–c-Myc (sc-789) and mouse anti-phosphorylated ERK (pERK; sc-7383; Santa Cruz); mouse anti-avian Src (clone EC10) and mouse anti-cortactin (clone 4F11; Upstate); goat anti-uPAR (AB8903) and mouse anti–MT1-MMP (MAB 3317; Chemicon International); mouse anti–MMP-2 (Ab-4; Oncogene Research Products); rabbit anti-pY421 cortactin (44-854G; BioSource) and mouse anti–MMP-9 (Ab-10; Calbiochem). Second antibodies conjugated to Alexa 488 or Alexa 350 were from Molecular Probes. Fluorescence images were acquired using a Zeiss Axiovert S100 microscope equipped with a Plan-Neofluor 40× objective and a Cooke SensiCam or with a Leica TCS-SP2 RS confocal laser-scanning microscope using a PL APO 100 1.40 oil immersion objective. Stacks of images were reconstructed in the z-dimension using Leica Confocal Software Lite Version.

Results

VSMCs Cultured on Glass Coverslips Form Podosomes in Response to Active Src

Primary rat aorta VSMCs cultured on glass coverslips contained prominent actin stress fibers and large vinculin-
containing focal adhesions (Figure 1A). On expression of activated Src-Y527F, VSMCs lost most of their stress fibers and focal adhesions and generated numerous dot-like actin-rich structures (diameter of 0.5 to 2 μm) (Figure 1B, upper panel). In vertical sections the actin structures appeared as columns that extended from the ventral cell surface upwards into the cytoplasm (Figure 1B, lower panels). The actin-rich columns colocalized with markers for podosomes, including cortactin (Figure 1B, lower panels) and tyrosine-phosphorylated proteins (Figure 2).\(^5\) Vinculin partially redistributed to the actin-rich dots but primarily remained associated with focal adhesions near the cell periphery (Figure 2). Both the urokinase-type plasminogen receptor (uPAR) and the matrix metalloproteinase MMP-9 were enriched at the actin dots, whereas MMP-2 exhibited a diffuse distribution (Figure 2).

The actin-rich structures formed by VSMCs in response to the expression of Src-Y527F therefore have a morphology and composition consistent with their identification as podosomes.

**VSMCs Expressing Src-Y527F Form ECM-Degrading Invadopodia**

To examine the ability of VSMCs to degrade the ECM, cells were cultured on FITC-fibronectin attached to a layer of cross-linked gelatin.\(^23\) In this assay proteolysis of the FITC-fibronectin results in the appearance of dark nonfluorescent areas. Only about 5% of control primary VSMCs produced tiny dot-like nonfluorescent spots (average size \(\approx 10 \, \mu m^2\)) after 72 hours (Figure 3A). In contrast, 95±5% of VSMCs expressing Src-Y527F were associated with areas of ECM digestion (average digested area per cell of 650±120 \(\mu m^2\)) by 48 hours after transfection (Figure 3B). The sites of ECM digestion colocalized with dot-like actin-rich structures that often clustered together to form arcs or rings (Figure 3B, arrow, upper, and middle panels). By 72 hours after transfection VSMCs expressing Src-Y527F had adopted a polarized migratory phenotype characterized by the presence of an actin-rich leading edge (Figure 3B, lower panel). Extensive areas of ECM degradation that were not associated with the VSMCs were visible, and can be attributed to the fact that the Src-Y527F–expressing VSMCs are able to break cell-substrate contacts and exhibit random motility. Vertical sections showed that the actin-rich structures were protrusions from the ventral surface that penetrated through the FITC-fibronectin layer into the cross-linked gelatin matrix (Figure 3C). The actin-rich protrusions colocalized with cortactin (data not shown), tyrosine-phosphorylated cortactin, and MT1-MMP (Figure 3D). The results suggest that the podosomes formed by Src-Y527F–expressing VSMCs cultured on glass are converted into ECM-degrading protrusions, similar to invadopodia, when cells are cultured on an ECM.

**A7r5 Cells and VSMCs Respond Differently to PDBu and Src-Y527F**

A7r5 cells cultured on FITC-fibronectin rapidly formed podosomes when treated with 1 μmol/L PDBu but showed little ability to digest the ECM (Figure 4A, upper panels). Expression of Src-Y527F caused A7r5 cells to form podosomes that clustered together into large rings but, again, digestion of the FITC-fibronectin was not observed (Figure 4A, lower and middle panels). Primary VSMCs did not respond to 1 μmol/L PDBu, either by rearranging the actin cytoskeleton or by digesting the FITC-fibronectin matrix (results not shown). A 10-fold higher concentration of PDBu caused 5% to 10% of VSMCs to disassemble stress fibers, form punctate actin-rich structures, and degrade large areas (average of 1900 \(\mu m^2\) per cell) of FITC-fibronectin in a diffuse manner (Figure 4B).

**Invadopodia Formation Is Promoted by Cdc42 and Rac**

Studies were undertaken to investigate the signaling pathways required for primary VSMCs to form invadopodia. Expression of active Cdc42V12 caused VSMCs to form
numerous large invadopodia that penetrated 4 to 5 μm into the ECM (Figure 5). VSMCs expressing active Rac1L61 extended broad lamellipodia and became more rounded but retained a well organized array of actin stress fibers (Figure 5). These cells produced elongated patches of FITC-fibronectin digestion that paralleled the stress fiber array. Cdc42V12 was nearly as effective as Src-Y527F, and more than twice as effective as Rac1L61, in promoting the formation of invadopodia that digested the ECM (Figure 6A). The invadopodia formed by VSMCs transfected with Src-Y527F were more aggressive than those formed by cells expressing Cdc42V12 or Rac1L61 as quantified by the area of FITC-fibronectin digested per cell (Figure 6B). The coexpression of dominant negative Cdc42N17 or Rac1N17 significantly increased larger areas of digestion at 48 hours (arrows). C, VSMCs transfected with Src-Y527F were cultured on FITC-fibronectin for 48 hours, stained with TRITC-phalloidin, and imaged using a confocal microscope. The lower panels show an x−z profile through the dashed line shown in the upper panels. V indicates ventral cell membrane, and B the bottom. Optical sections were taken every 0.5 μm. D, VSMCs transfected with Src-Y527F were cultured on FITC-fibronectin for 48 hours, stained with TRITC-phalloidin, and imaged using a confocal microscope. The lower panels show an x−z profile through the dashed line shown in the upper panels. V indicates ventral cell membrane, and B the bottom. Optical sections were taken every 0.5 μm.

Figure 3. Primary VSMCs expressing Src-Y527F form invadopodia-like protrusions that digest the ECM. VSMCs were cultured on a matrix consisting of FITC-fibronectin on top of cross-linked gelatin. Proteolysis of the FITC-fibronectin (FITC-Fn) results in the appearance of black nonfluorescent regions. A, Control VSMCs cultured for 48 or 72 hours on FITC-fibronectin were stained with TRITC-phalloidin. Tiny regions of ECM degradation were sometimes visible at 72 hours (arrows). Merged images (overlay) show staining for actin (red) and FITC-fibronectin (green). B, VSMCs transfected with chicken Src-Y527F were cultured on FITC-fibronectin for 24 to 72 hours and stained with TRITC-phalloidin. Cells expressing Src-Y527F, identified using anti-avian Src (Src), contained numerous actin-rich dots, rings, and arcs and actively digested the FITC-fibronectin. Merged images show that actin-rich structures (red) localized to areas of FITC-fibronectin degradation at 24 hours and outlined larger areas of digestion at 48 hours (arrows).
Habited the ability of Src-Y527F to promote invadopodia formation as measured by the percent of VSMCs that digested the FITC-fibronectin and the area of FITC-fibronectin digested per cell (Figure 6A and 6B). Treatment of Src-Y527F–transfected VSMCs with U0126, a potent inhibitor of the ERK activators MEK1 and MEK2, virtually eliminated digestion of the ECM (Figure 6A and 6B). Moreover, an antibody against active phosphorylated ERK (pERK) labeled the area surrounding the actin-rich podosome core in Src-Y527F–expressing VSMCs (Figure 2). A selective inhibitor of the p38 MAPK pathway, SB203580, had no effect on ECM digestion induced by Src-Y527F (Figure 6A and 6B). Immunoblot analysis using an anti-phospho antibody to MK2, a p38 MAPK substrate, demonstrated that SB203580 was effective in suppressing p38 MAPK activity in the VSMCs (supplemental Figure I).

**PAK1 Induces Invadopodia Formation in a Kinase-Independent Manner**

Overexpression of wild-type PAK1, a key downstream target of Cdc42 and Rac1, had no significant effect on the VSMC actin cytoskeleton and did not induce ECM digestion (data not shown). PAK1-LL, a PAK1 mutant that exhibits impaired binding to Cdc42/Rac and displays a relatively high level of constitutive kinase activity, caused VSMCs to extend large polarized lamellipodia but only weakly promoted invadopodia formation (Figures 5 and 7). Interestingly, a kinase-inactive version of PAK1-LL (PAK1-LL/R) was highly effective in inducing invadopodia formation in VSMCs. More than 80% of VSMCs expressing PAK1-LL/R developed large invadopodia that colocalized with extensive regions of focal ECM digestion (Figures 5 and 7). This finding focused attention on the protein–protein interactions mediated by PAK1. In A7r5 cells, the ability of PAK to induce podosomes depends on its interaction with the guanine nucleotide exchange factor PIX. Similarly, a PAK1-LL/R mutant in which the PIX binding site was eliminated exhibited a severely reduced ability to induce the formation of ECM-degrading invadopodia in VSMCs (Figure 7A and 7B).

Addition of the potent Src inhibitor SU6656 or coexpression of a kinase-dead dominant negative Src-K295R,Y527F mutant severely impaired the ability of PAK1-LL/R to induce assembly of invadopodia (Figure 7A and 7B). Activation of the ERK MAP kinase pathway, but not the p38 MAP kinase pathway, was also required for invadopodia formation by PAK1-LL/R (Figure 7A and 7B). The protein kinase C inhibitor, BIM I, had no effect on ECM digestion induced by PAK1-LL/R (Figure 7A and 7B).

**Formation of Invadopodia by VSMCs in a 3-D Collagen Culture**

Synthetic VSMCs in vivo must maneuver through a 3-D ECM. To assess the ability of primary VSMCs to generate...
invadopodia under these more physiologically relevant conditions, cells were embedded within a 3-D matrix of collagen for 24 hours, then were fixed and stained for actin and cortactin. 3-D reconstructions of confocal sections showed that the primary VSMCs adopt a spindle-shaped phenotype (Figure 8A). Examination of VSMCs migrating out of a central aggregate indicate that the long pseudopodia (at the right in Figure 8A) represent the leading edge. Strikingly, a number of small actin- and cortactin-rich protrusions (average of 8±3 per cell) were present on the surface of the VSMCs. These protrusions were similar in size to the invadopodia formed by Src-Y527F–expressing VSMCs on a 2-D matrix.

To examine whether the protrusions were involved in digestion of the ECM, VSMCs were embedded in a 3-D matrix of FITC-labeled collagen. Confocal images showed the presence of large cellular extensions that penetrated through holes in the collagen matrix (supplemental Videos I to IV). It seems plausible that digestion of the ECM is performed initially by small invadopodia such as those observed in VSMCs cultured on a 2-D ECM. As digestion proceeds, though, the invadopodia turn into much larger actin- and cortactin-rich extensions such as are shown in Figure 8 and the supplemental videos.

If the protrusions extended by VSMCs embedded in a 3-D collagen matrix are related to invadopodia, it would be predicted that the expression of PAK1-LL/R would promote production of these protrusions. Indeed, when VSMCs expressing PAK1-LL/R were cultured within a 3-D collagen matrix they exhibited a nonpolarized phenotype characterized by an increased number, relative to control VSMCs, of large cellular extensions and small actin- and cortactin-rich protrusions (average of 14±3 per cell) (Figure 8B). This result lends support to the view that the protrusions present on VSMCs cultured within a 3-D collagen matrix are related to invadopodia and that they play a role in digestion of the matrix to facilitate cell migration.

**Discussion**

Invasion of the intimal layer of damaged arteries by VSMCs contributes to a variety of vascular diseases, including post-

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**Figure 6.** Src-Y527F–induced invadopodia formation is dependent on Cdc42, Rac, and MEK/ERK. VSMCs cultured on FITC-fibronectin were transfected with Cdc42V12, Rac1L61, or Src-Y527F. Cells transfected with Src-Y527F were cotransfected with Cdc42N17 or RacN17 or were treated with the MEK1/2 inhibitor U0126 (50 μmol/L) or the p38 MAPK inhibitor SB203580 (10 μmol/L) as labeled. A, The percent of transfected VSMCs associated with visible areas of FITC-fibronectin digestion was determined after 48 hours. Values were obtained by examining 200 cells in each of 3 separate experiments. B, For each VSMC that digested the ECM the area of digestion was measured using ImagePro Plus 5.0 software. Measurements were made for 20 to 30 cells in each of 3 separate experiments. Error bars indicate the standard deviation.

**Figure 7.** Invadopodia formation induced by PAK1-LL/R is dependent on PIX, Src, and MEK/ERK. VSMCs cultured on a matrix consisting of FITC-fibronectin on top of a layer of cross-linked gelatin were transfected with PAK1-LL, PAK1-LL/R, or with a PIX binding mutant of PAK1-LL/R (PIX-). Cells transfected with PAK1-LL/R were cotransfected with dominant negative Src or treated with the Src inhibitor SU6656 (1 μmol/L), the MEK inhibitor U0126 (50 μmol/L), the p38 MAPK inhibitor SB203580 (10 μmol/L), or the PKC inhibitor BIM I (1 μmol/L) as labeled. A, The percent of transfected VSMCs associated with visible areas of FITC-digestion was determined after 48 hours. Values were obtained by examining 200 cells in each of 3 separate experiments. B, For each VSMC that digested the ECM the area of digestion was measured. Measurements were made for 20 to 30 cells in each of 3 separate experiments. Error bars indicate the standard deviation.
On a glass substrate VSMCs expressing Src-Y527F formed a layer of fibronectin on top of a cross-linked gelatin matrix. They were cultured on a glass surface or an ECM consisting of collagen matrices VSMCs spontaneously extended protrusions that digested holes in the ECM.

Collagen matrices VSMCs spontaneously extended protrusions that digested holes in the ECM. VSMC podosomes also contained uPAR, which enhances VSMC invasion by catalyzing the conversion of plasminogen to plasmin.30 The expression of activated Src-Y527F caused VSMCs to form 2 different types of structures, depending on whether primary rat aorta VSMCs are capable of forming ECM-degrading podosomes and invadopodia, structures that play a central role in tissue invasion by cells. In 2-D cultures, the formation of invadopodia by VSMCs was dependent on the expression of activated Src, Cdc42, or PAK1, but in 3-D collagen matrices VSMCs spontaneously extended protrusions that digested holes in the ECM.

The expression of activated Src-Y527F caused VSMCs to form 2 different types of structures, depending on whether they were cultured on a glass surface or an ECM consisting of a layer of fibronectin on top of a cross-linked gelatin matrix. On a glass substrate VSMCs expressing Src-Y527F formed numerous small columns of filamentous actin that projected upwards from the bottom of the cell into the cytoplasm (Figure 1B). These structures are morphologically similar to the podosomes generated by A7r5 cells in response to PDBu (Figure 1B).14–16 The actin columns produced by Src-Y527F-expressing VSMCs colocalized with markers for podosomes, including cortactin, an SH3 domain-containing actin binding protein that stimulates the actin-nucleating activity of the Arp2/3 complex (Figure 2).24 The recruitment of cortactin is an early and essential step in the formation of podosomes by PDBu-treated A7r5 cells and it likely plays a similar role in VSMCs.25, 26 A number of different ECM-degrading MMPs are concentrated at podosomes formed by osteoclasts and subconfluent endothelial cells.5,27 The podosome-like structures formed by VSMCs were enriched in MMP-9, a gelatinase that is upregulated after injury to rat carotid arteries and that promotes VSMC invasion (Figure 2).28 In contrast, MMP-2, a related gelatinase that is constitutively expressed in cultured human VSMCs, did not localize to the podosomes, although it too is implicated in VSMC invasion. The hyaluronan receptor CD44 assists in the attachment of MMP-9 to the surface of VSMCs and recruits MMP-9 to invadopodia formed by breast cancer cells.28,29 It will be interesting to determine whether CD44 accounts for the differential localization of MMP-2 and MMP-9 to VSMC podosomes. VSMC podosomes also contained uPAR, which enhances VSMC invasion by catalyzing the conversion of plasminogen to plasmin.30

When VSMCs expressing Src-Y527F were cultured on the fibronectin-gelatin ECM they extended actin-rich protrusions that strongly resembled the invadopodia formed by highly invasive cancer cells.4,31 The protrusions formed by the VSMCs correlated with sites of ECM digestion and contained MT1-MMP, a key enzyme responsible for gelatin matrix digestion by breast carcinoma cell invadopodia.32 MT1-MMP is essential for the ability of VSMCs to degrade and infiltrate 3-D barriers of interstitial collagen, suggesting that its localization to invadopodia may be of particular importance for VSMC invasion.33 The protrusions formed by Src-Y527F-expressing VSMCs were also enriched in tyrosine phosphorylated cortactin, a form of cortactin that is preferentially localized to breast cancer cell invadopodia.7 It seems reasonable to conclude that the podosomes formed by Src-Y527F-expressing VSMCs cultured on glass are transformed into invadopodia when the cells are placed on a digestible gelatin-fibronectin matrix. These results support the concept that VSMCs, like other invasive cells, use invadopodia to focus a variety of proteolytic activities and to efficiently remove ECM barriers to migration.

The results presented in Figure 4A indicate that the podosomes formed by A7r5 cells, whether in response to PDBu or transfection with Src-Y527F, exhibit only a very limited ability to digest the fibronectin matrix (Figure 4). This is in contrast to a study showing that PDBu-treated A7r5 cells locally degrade fibronectin in the vicinity of podosomes.34 However, in the latter study the fibronectin layer was coated directly onto glass coverslips and was subject to extensive rearrangement by A7r5 cell contractile activity. Thus, loss of fibronectin at and around the podosomes could have resulted either from digestion or contractile forces. The conclusion...
that primary VSMCs represent a more aggressively invasive cell type than A7r5 cells is also supported by the observation that A7r5 cells are unable to degrade chemically cross-linked fibronectin or gelatin, whereas VSMCs extended invadopodia into the layer of cross-linked gelatin (eg, Figure 5). The reason for the more aggressive invasive behavior of VSMCs requires further investigation. Possibly, A7r5 cells have a reduced ability to synthesize or secrete proteases, lack cell surface receptors required to localize the proteases, or are missing cytoskeletal components needed to stabilize protrusive invadopodia. VSMCs differed from A7r5 cells in being much less responsive to PDBu. Phorbol esters stimulate podosome formation in A7r5 cells and in endothelial cells by activating PKCa and PKCθ, which lie upstream of Src. Possible candidates include AFAP-110 and the protein tyrosine phosphatase PTPζ, which are involved in the activation Src by PKCα and PKCθ, respectively.

Invadopodia formation in VSMCs could be potently induced by active Cdc42 and Rac1 and by a kinase-dead version of the Cdc42/Rac1 effector PAK1 (Figure 5). As in A7r5 cells, the ability of kinase-dead PAK1-LL/R to induce invadopodia was dependent on its ability to bind PIX, a guanine nucleotide exchange factor that can feed back to activate Cdc42 and Rac. SRC and ERK kinase activity were also necessary for PAK1-LL/R to promote invadopodia formation. The requirement for ERK is of interest, because PAK1 directly binds ERK and facilitates its activation in VSMCs. Whether a direct interaction of PAK1 with ERK is involved in invadopodia formation is under investigation. The constitutively active PAK1-LL only weakly promoted invadopodia formation, suggesting that its ability to phosphorylate substrates such as myosin light chain kinase (MLCK), LIM kinase, paxillin, and vimentin, may actively repress invadopodia formation.

VSMCs and A7r5 cells adopt an elongated spindle-shaped morphology when placed in a 3-D ECM. Confocal microscopy revealed that the elongated VSMCs contained numerous small actin- and cortactin-rich protrusions on their surface that appear morphologically similar to invadopodia (Figure 8). A relationship to invadopodia was also indicated by the finding that the number of protrusions is increased in cells expressing PAK1-LL/R. Interestingly, spindle-shaped A7r5 cells cultured in a 3-D matrix of Matrigel also display numerous small actin-rich surface protrusions, which appear similar to the peripheral podosomes that form following the addition of PDBu (see Figure 3, Ref. 34). It is well established that the ECM plays a major role in modulating VSMC phenotype, morphology, and motility. Possibly, the engagement of certain 3-D matrices upregulates signaling pathways, such as Src or PAK1, that promote invadopodia formation and invasive tunneling behavior (supplemental Videos I to IV).

In summary, the results presented here show that VSMCs are capable of producing invadopodia that digest the ECM and raises the possibility that the formation of these structures facilitates the movement of VSMCs through ECM barriers. Therapeutic control of invadopodia formation may provide significant advantages to other approaches in limiting injury-dependent vascular restructuring and occlusion.

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Disclosures

None.

References


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**Supplementary Videos**

Video Legend. The videos show a stack of 29 optical sections taken at 0.5 μm intervals of VSMCs embedded in a 3-D matrix of FITC-collagen type I and stained for actin, cortactin and nuclear DNA. Video 1 shows actin (magenta), video 2 shows FITC-collagen (green), video 3 shows cortactin (red) and video 4 shows a merged image of actin, cortactin, FITC-collagen and DAPI (blue). A schematic view of the cell shown in the videos is presented below. The first optical section shows the tip of an invadopodium that has penetrated through the collagen matrix. Subsequent sections, moving upwards, show the cell body and nucleus. Cortactin is enriched at the tip of cellular extensions, including the invadopodia. Below the schematic diagram are shown cropped images from the first optical section. The arrow indicates the position of dark regions in the collagen matrix that appear to be holes digested in the matrix by the invadopodium.

Methods: VSMCs were embedded in a 3D matrix of collagen as described in "Materials and Methods" except that a 2 mg/ml solution of collagen composed of 1 mg/ml bovine skin FITC-conjugated collagen type I (Sigma) and 1 mg/ml rat tail collagen type I was used. After 24 hours in DMEM with 20% FBS, cells were fixed and stained with TRITC-phalloidin, DAPI and a mouse anti-cortactin antibody. An Alexa 633 conjugated goat anti-mouse second antibody was used. Confocal sections were taken using a Leica TCS-SP2 RS confocal laser-scanning microscope and converted into a video using Leica Confocal Software Lite Version.
Fig. 1. Inhibition of p38 MAPK activity by SB203580 in VSMCs. VSMCs were starved for 48 h in DMEM containing 0.1% FBS, 1 % penicillin-streptomycin and, for the final 24 h, the indicated concentration of SB203580. VSMCs were stimulated with (A) 20 ng/ml TNF-α for 15 min or (B) 20 ng/ml PDGF-BB for 5 min and then subjected to immunoblot analysis using an anti-phospho antibody to MK2 (anti-pMK2, Cell Signaling), a p38 MAPK substrate. Blots were stripped and reprobed using an antibody to p38 MAP kinase. The results show that SB203580 at concentrations of 1 µM or higher block p38 MAPK-mediated phosphorylation of MK2. Immunoblots were visualized using a Horse Radish Peroxidase coupled second antibody and the ChemiLucent ECL detection system (Millipore).