Thrombin Stimulates Smooth Muscle Cell Differentiation From Peripheral Blood Mononuclear Cells via Protease-Activated Receptor-1, RhoA, and Myocardin

Kenneth Martin,* Sharon Weiss,* Pat Metharom, Jeffrey Schmeckpeper, Brian Hynes, John O’Sullivan, Noel Caplice

Rationale: Smooth muscle precursor cells have previously been reported to reside in bone marrow and in the circulation, but little is currently known regarding the proximate stimuli for smooth muscle cell differentiation of these putative progenitors.

Objective: Because local thrombin generation occurs as an initial response to vascular injury, we hypothesized that thrombin may influence the differentiation of circulating smooth muscle progenitor cells.

Methods and Results: Peripheral blood mononuclear cells were cultured on type I collagen using a protocol optimized to stimulate smooth muscle cell outgrowth. Thrombin-stimulated upregulation of the transcription factor myocardin and smooth muscle myosin heavy chain, and both were inhibited by hirudin or the RhoA inhibitor Y27632. After 10 days of culture, smooth muscle outgrowth colonies formed, which stained positive for α-smooth muscle actin, smooth muscle myosin heavy chain, and calponin, in addition to having a contractile response to 100 nmol/L angiotensin II. Coincubation of peripheral blood mononuclear cells with thrombin, 10 μmol/L protease-activated receptor-1, but not protease-activated receptor-4 activating peptide significantly increased the number of smooth muscle outgrowth colonies formed. Thrombin-induced enhancement of smooth muscle outgrowth colony formation was inhibited by hirudin, Y27632, and an antibody against protease-activated receptor-1.

Conclusions: These data illustrate a novel thrombin-induced pathway for smooth muscle differentiation from putative smooth muscle progenitors in peripheral blood.

We have previously described circulating smooth muscle outgrowth cells (SOCs).1,2 Moreover, we have shown in human subjects who have undergone bone marrow transplantation, smooth muscle cells (SMCs) of donor origin are markedly enriched in coronary atherosclerotic plaque compared to the nondiseased vessel wall.3 A number of potential agents may mediate SMC differentiation from blood borne progenitors including transforming growth factor-β; platelet-derived growth factor-BB; and sphingosine-1-phosphate, which induces RhoA-dependent myocardin expression and SMC differentiation in mesenchymal stem cells.4 Thrombin generation occurs at sites of injury and could conceivably contribute to smooth muscle differentiation of circulating progenitor cells based on several lines of evidence. Under various in vitro conditions in mature SMCs, thrombin stimulates upregulation of the SMC marker genes smooth muscle myosin heavy chain (SM-MHC)6 and calponin.7 Thrombin also enhances the angiogenic potential of endothelial progenitor cells8 and may influence vascular progenitor phenotype and function.9 In the context of circulating progenitors, no study to date has fully investigated the downstream effects of thrombin on circulating mononuclear cells. Here, we show that thrombin induces robust sequential upregulation of the master smooth muscle cell transcription factor myocardin, as well as smooth muscle specific protein expression in peripheral blood mononuclear cells (PBMCNs). Moreover, these transcriptional events precede marked augmentation of colony outgrowth of cells with morphological and ultrastructural characteristics of smooth muscle cells and full contractile competence. We further characterize the signaling events leading to SMC marker transcription and formation of contractile SOCs, linking thrombin activation of PAR-1 and myocardin upregulation via the RhoA pathway.

Methods

PBMCNs were isolated from blood using Biocoll density gradients and cultured using a protocol optimized for smooth muscle cell outgrowth. Details of the experimental procedures used in this study are provided in the Online Data Supplement at http://circres.ahajournals.org. All data presented represent at least 4 to 5 individual experiments performed in triplicate.

Results and Discussion

Thrombin Induces Myocardin and Smooth Muscle Myosin Heavy Chain Protein Expression in PBMCNs via RhoA

Because myocardin is recognized as a master transcription factor for smooth muscle–specific gene expression, the effect of thrombin on myocardin and SM-MHC expression in cultured PBMCNs was initially investigated. Myocardin and SM-MHC upregulation by thrombin peaked at three and 5 days (Figure 1A) after initial seeding culture, and protein levels were, respectively, 3- and 2-fold greater than control treatment (P<0.05; Figure 1B and 1C). Downregulation of SM-MHC expression observed from day 7 may be indicative of the phenotype modulation before SOC colony expansion (burst), which is characteristic of adult SMCs, whereby proliferating cells downregulate their structural proteins (reviewed elsewhere10). Thrombin-induced upregulation of myocardin and SM-MHC (Figure 1D) was blocked by hirudin and Y27632, a RhoA inhibitor (P<0.001; Figure 1E and
Normalization of myocardin and SM-MHC antigen to proliferating-cell nuclear antigen (PCNA) expression and proliferation indices (5-bromodeoxyuridine incorporation; Online Figure II) in thrombin and untreated groups indicated that thrombin-stimulated myocardin upregulation was attributable to a differentiation rather than a proliferative stimulus. Myocardin associates with serum response factor, inducing CArG SMC gene transcription. Most contractile SMC marker genes such as α-smooth muscle actin (α-SMA), SM-MHC, and calponin contain at least 1 CArG element located within the promoter–enhancer region of the gene. These data suggest, for the first time, a link between thrombin/RhoA signaling, myocardin expression, and smooth muscle cell differentiation.

Thrombin-Induced SOC Colony Formation Is Inhibited by Hirudin and RhoA Blockade

To explore further whether thrombin could augment smooth muscle differentiation of PBMNCs, extended culture of these cells was performed under smooth muscle permissive conditions in the presence or absence of thrombin. After 10 days of culture, SOC colonies with characteristic “hill and valley” morphology that stained positive for α-SMA were evident (Figure 2A). Colony number was increased ~2.5-fold by 1 U/mL thrombin compared to control treatment (*P<0.01; Figure 2B). Both hirudin and Y27632 significantly inhibited thrombin-induced augmentation of SOC outgrowth from PBMNCs (Figure 2B). Hirudin or Y27632 administered in the absence of thrombin had no significant effect on SOC colony formation (Figure 2B). Flow cytometric analysis of SOCs revealed a SMC protein expression profile similar to mature aortic (Ao)SMCs, with cells staining positive for the SMC markers, α-SMA, SM-MHC, and calponin but staining negative for endothelial nitric oxide synthase (Figure 2C). SOCs had contractile activity, in response to 100 nmol/L angiotensin II (20.8±2.1% contraction), comparable to that of AoSMCs (14.9±3.3, versus SOCs; P=NS; Figure 2D). The calcium channel blocker nifedipine inhibited contraction of both SOCs (−0.5±3.4%, P<0.001) and AoSMCs (−3.1±2.7%, P<0.001) equally. Ultrastructural analysis of SOCs revealed a myofilamentous staining pattern for α-SMA and calponin (Figure 2E) similar to contractile AoSMCs. Taken together, these results indicate that thrombin promotes the formation of SOCs from peripheral blood, which possess the capacity to form functional SMCs.

PAR-1, but Not PAR-4 Activation, Enhances SOC Colony Formation From the PBMNCs

Because PAR receptors on PBMNCs are the most likely to mediate thrombin signaling, RT-PCR analysis was performed and confirmed strong expression of PAR-1 but not PAR-4 receptor on these cells (Figure 3A). Indeed, 10 μmol/L PAR-1 activating peptide12 but not PAR-4AP significantly stimulated SOC colony formation (≈2-fold increase in SOC colony formation units versus control treatment, P<0.01;
Figure 2. SOC colony formation stimulated by thrombin. A, SOC colony with a characteristic central rosette and “hill and valley” morphology formed from cultured PBMNCs and stained positive for α-SMA. Scale bar represents 200 μm. B, Thrombin induced an ~2.5-fold increase in SOC colony formation (*P<0.05 compared to control treatment). Control-treated PBMNC cultures, those with 30 U/mL hirudin alone or 10 μmol/L Y27632, showed no significant difference in SOC colony numbers. Thrombin augmentation of SOC colony formation was inhibited by hirudin or Y27632 (*P<0.05, compared to thrombin treatment). C, Flow cytometric analyses showing that SOCs derived from PBMNCs stained positive for SM-MHC, α-SMA, and calponin but not endothelial nitric oxide synthase (eNOS). AoSMCs also stained positive for SMC markers, but freshly isolated PBMNCs were negative for all markers. D, SOCs and AoSMCs contract similarly in response to 10 nmol/L angiotensin II over 5 minutes in a single-cell contraction assay. There was no contraction response in PBMNCs (*P<0.01 vs SOC). Contraction responses of SOCs and AoSMCs were completely inhibited by 1 μmol/L nifedipine (*P<0.01 vs no nifedipine). Representative images of contraction in response to angiotensin II are shown for PBMNCs and SOCs. E, Immunofluorescence staining of SOCs showing myofilamentous organization (red) of α-SMA, SM-MHC, and calponin. Isotype control (IgG) antibody shows no staining. Nuclei counterstained on each image with DAPI (blue). Scale bar represents 50 μm.
Figure 3B). This increase was similar to thrombin-induced enhancement, suggesting that thrombin may act via PAR-1 to stimulate SOC colony formation in PBMNCs. PAR1-AP–induced augmentation was completely inhibited by Y27632 (P<0.05) but not by hirudin (Figure 3C), which is unsurprising because activation of PAR-1 using the tethered ligand peptide is insensitive to hirudin. However, an antibody raised against the N terminus of PAR-1, which includes the...
thrombin cleavage and tethered ligand sequences inhibited both thrombin-induced SOC colony formation stimulation (P<0.01; Figure 3D) and SM-MHC expression (P<0.05; Figure 3E).

These data provide evidence for thrombin as a differentiation factor for putative smooth muscle progenitor cells within peripheral blood. Moreover, a PAR-1–dependent SMC differentiation pathway linking RhoA signaling, myocardin upregulation, and smooth muscle–specific protein expression (Figure 3F) may have implications for understanding how coagulation proteases contribute to cell fate in the context of thrombosis and mononuclear cell infiltration of the injured vessel wall.

Acknowledgments
We thank Sanja Trinki for graphic design assistance.

Sources of Funding
This work was supported by grants from the Health Research Board (Ireland) and Science Foundation Ireland.

Disclosures
None.

References

KEY WORDS: thrombin ■ RhoA ■ myocardin ■ smooth muscle differentiation ■ circulating progenitor cells
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Circ Res. 2009;105:214-218; originally published online July 2, 2009;
doi: 10.1161/CIRCRESAHA.109.199984

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/3/214

Data Supplement (unedited) at:
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Thrombin Stimulates Smooth Muscle Cell differentiation from Peripheral Blood Mononuclear Cells via PAR-1, RhoA, and Myocardin

Materials and Methods

Cell culture
Blood was collected from Landrace pigs into EDTA-anticoagulated vacutainers. Mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrom, Germany) as previously described\(^1\). Red blood cells were lysed using ACK lysis buffer (0.01 M KHCO\(_3\), 0.02 M NH\(_4\)Cl, 0.01 mM EDTA) and the remaining MNCs were subsequently washed three times with MCDB\(^{+++}\) (MCDB media supplemented with 1 \(\mu\)g/ml hydrocortisone acetate, 0.5 mM dibutyrl cAMP, 0.25 \(\mu\)g/ml amphotericin B, 10 ng/ml VEGF, 6.7 U/ml heparin, 100 U/ml penicillin/streptomycin, 1.6 mM L-glutamine) and counted. Cells were plated on type I collagen coated 6-well plates at a density of 2x10\(^7\) cells/well in EGM-2 culture media in the presence or absence of 1 U/ml thrombin (and other specified agents). Cells were cultured over 10 days until colonies started forming. The colonies were counted for each treatment. Porcine aortic smooth muscle cells (AoSMCs) used as positive control cells were isolated as previously described\(^2\).

Immunoblotting
Expression of myocardin, PCNA and SMMHC markers was evaluated by Western blot analysis. For nuclear proteins (myocardin and PCNA) cells were washed, scraped in PBS and pelleted by centrifugation (14,000 x g, 10 mins, 4°C). 60 \(\mu\)l of “crack” buffer (50 mM Tris-HCl, pH 6.8; 100 mM DTT; 1 mM sodium orthovanadate; 100 \(\mu\)g/ml PMSF;
2% SDS; 10% glycerol; supplemented with Complete protease inhibitors [Roche]) was added to each pellet. The lysate was sheared through a 23-gauge needle, boiled for 10 min, and then spun at 4°C for 10 min at 14,000 × g. The protein in the supernatant was quantified using Bradford Reagent (Sigma), resolved by 10% SDS-PAGE, before immunoblotting using primary antibodies against myocardin (1:100 M-16, Santa Cruz) or PCNA (1:250; Chemicon). Bands quantified using Image J (NIH software). Myocardin band intensity was normalised to PCNA band intensity.

For total cellular protein extraction cells scraped from each well were resuspended in 60 µl of “Freeze-Thaw” buffer (600 mM KCl; 20 mM Tris-Cl (pH 7.8); 20% Glycerol; supplemented with Complete protease inhibitors [Roche]). Cells were snap frozen in liquid nitrogen, and then thawed on ice for a total of 3 cycles. Protein concentration was quantified using Bradford Reagent (Sigma), resolved by 12% SDS-PAGE, and detected by immunoblotting using a 1:1000 dilution of anti-β-actin (Sigma), 1:200 SM-MHC (Dako, Carpinteria, USA) primary antibodies and appropriate secondary peroxidase-conjugated antibodies and SuperSignal chemiluminescence (Pierce).

**RhoA activation Assay**

RhoA activation was assessed using a commercial RhoA activation assay kit (Millipore). In brief, PBMNCs were stimulated by thrombin for the described time and then lysed (Mg²⁺ lysis/washing buffer). The lysates were incubated with GST-tagged Rhotekin Rho-binding domain. The bound protein was pulled-down and resolved on 12% SDS-PAGE. The gel was transferred onto nitrocellulose membrane and detected by immunoblotting using 1:250 anti RhoA primary antibody (clone 55, Millipore) and the appropriate peroxidise-conjugated secondary antibody and Immobilon chemiluminescence (Millipore).

**RT-PCR**

Total RNA was extracted from freshly isolated PBMNCs using SV total RNA isolation system (Promega). 2-5 µg of total RNA was subjected to cDNA synthesis with 200 U SuperScript III RT (Invitrogen), and 500 ng oligo(dT). 2 µl of the synthesised cDNA were amplified using specific primers with Platinum Taq polymerase (Invitrogen). PCR
products were resolved on 2% agarose gels at 90V for 45min and visualized on an UV transilluminator.

Primers used:

<table>
<thead>
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<th>Target</th>
<th>Primer Sequences</th>
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<tr>
<td></td>
<td>ctagttgccccagagatgc (R)</td>
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**Single cell contraction**

Cells cultured on glass bottom plates (MaTek corp.) were washed twice with KHB. Contraction assays were performed in the presence or absence of nifedipine (1 µM). Snap shots of the imaged cells were acquired before and every minute (up to 5min) after addition of 100 nM Angiotensin II. Analysis was performed using NIS-Elements BR 2.30 (Nikon) software to estimate the cell surface area. The change in cell surface area following addition of 100 nM Angiotensin II was calculated as percent contraction.

**Flow Cytometric Analysis of SMC Markers**

The expression of smooth muscle specific markers was analyzed by FACS using intracellular antigen immunolabeling protocol. Fresh PBMNCs, cultured SOCs and AoSMCs (detached with Versene) were washed in PBS. Cells were fixed with 2% paraformaldehyde and then permeabilized in 0.2% Tween 20 solution. Subsequently, cells were incubated with a 1:250 dilution of either anti-SMA (1A4), SM-MHC (G4) or Calponin (CALP) all from Dako (Glostrup, Denmark) for 1 hour on ice and subsequently with 1:500 PE conjugated anti-mouse secondary Ab. Data were obtained with FACSCalibur (BD) and analysed with WinMdi software.
**In-vitro immunofluorescence**

SOCs were cultured on 8 well NUNC slides and serum starved prior to staining. The cells were fixed with 2% ice-cold paraformaldehyde, rinsed in methanol, washed with PBS, and blocked with 10% serum for 20min. Slides were stained with Abs against the following; αSMA (1:200 clone 1A4), Calponin (1: 200, clone CALP), SM-MHC (1:200 G4, all Santa Cruz) for 1 hour, washed and probed with species-specific secondary Abs from Molecular Probe (Invitrogen), Alexa Fluor 546 conjugates. Slides were washed and DNA stained with DAPI (Invitrogen) before confocal laser microscopy (Nikon eC1 plus).

**Bromo-deoxyuridine (BrdU) Uptake**

Measures of proliferative activity by BrdU uptake was assessed as previously described\(^3\). Briefly, PBMNC were cultured as above in the presence of 10 µM BrdU (Sigma). At the indicated timepoints, the media was removed, washed twice with PBS and fixed with 4% paraformaldehyde. The cells were incubated with 1N HCl on ice for 10 mins, then with 2N HCl for 10 mins at RT, and a further 20 mins at 37°C. Slides were neutralised with borate buffer (0.1M, pH 9.2) and then stained overnight with a 1:150 dilution of an anti-BrdU antibody (ICR1, rat monoclonal, Abcam). Antibody binding was visualised using goat-anti-rat 546 (Invitrogen) and confocal microscopy.

**Statistical Analyses**

All data presented represent at least four to five individual experiments performed in triplicate. Comparisons between groups were made using unpaired Student’s t-tests and statistical significance was determined as a p value < 0.05.
References


RhoA Activation in PBMNC in Response to 1 U/ml Thrombin

Online Figure I

RhoA activation was observed as soon as 5 min following application of thrombin.
Online Figure II  Absence of proliferative activity in PBMNCs stimulated by thrombin. A: No significant augmentation of proliferation was observed in PBMNC stimulated by thrombin compared to control treatment up to day 7 of culture on type I collagen as determined by BrdU incorporation. * represents p<0.001, ns: p>0.05. B: Nuclei of SOC outgrowth cells at day 7 of culture show BrdU uptake (red, left panel) whereas PBMNC in early culture (Day 1-5, right panel) show lower levels of BrdU uptake. Scale bar represents 200 µm.
Online Figure III: Blockade of PAR-1 inhibited thrombin-induced SM-MHC upregulation as illustrated by representative immunoblot.