Smooth Muscle Progenitor Cells in Human Blood

David Simper, MD; Paul G. Stalboerger, BS; Carmelo J. Panetta, MD; Shaohua Wang, MD; Noel M. Caplice, MD, PhD

Background—Recent animal data suggest that vascular smooth muscle cells within the neointima of the vessel wall may originate from bone marrow, providing indirect evidence for circulating smooth muscle progenitor cells (SPCs). Evidence for circulating SPCs in human subjects does not exist, and the mechanism whereby such putative SPCs may home to sites of plaque formation is presently not understood but is likely to involve expression of specific surface adhesion molecules, such as integrins. In this study, we aimed to culture smooth muscle outgrowth cells (SOCs) from SPCs in human peripheral blood and characterize surface integrin expression on these cells.

Methods and Results—Human mononuclear cells isolated from buffy coat were seeded on collagen type 1 matrix and outgrowth cells selected in endothelial growth medium (EGM-2) or EGM-2 and platelet-derived growth factor BB. Selection in platelet-derived growth factor BB–enriched medium caused rapid outgrowth and expansion of SOC to >40 population doublings in a 4-month period. These SOCs were positive for smooth muscle cell–specific α actin (αSMA), myosin heavy chain, and calponin on immunofluorescence and Western blotting and were also positive for CD34, Flt1, and Flk1 receptor but negative for Tie-2 receptor expression, suggesting a potential bone marrow angioblastic origin.

In contrast, endothelial outgrowth cells (EOCs) grown in EGM-2 alone and the initial MNC population were negative for these smooth muscle–specific markers. Integrin α6β1 expression by FACS and Western blotting was significantly increased in SOCs compared with EOCs, and this was confirmed by 8-fold greater adhesion of SOC to fibronectin (P<0.001), an effect that could be decreased using an α6β1 antibody. Finally, SOC showed a significantly greater in vitro proliferative potential compared with EOCs of similar passage (P<0.001).

Conclusions—This study demonstrates for the first time outgrowth of smooth muscle cells with a specific growth, adhesion, and integrin profile from putative SPC in human blood. These data have implications for our understanding of adult vascular smooth muscle cell differentiation, proliferation, and homing. (Circulation. 2002;106:1199–1204.)

Key Words: muscle, smooth ■ progenitor ■ blood cells

Vascular smooth muscle cell migration, proliferation, and matrix synthesis within the intima of medium-sized and large vessels is thought to play a major role in atherosclerosis development in adult human subjects. In the embryo, these vascular cells have a complex origin, with the first smooth muscle cells surrounding endothelial tubes being derived from transdifferentiated endothelium during nascent vascular and cardiac valve development. Several growth factors have been implicated in embryonic smooth muscle cell differentiation, including transforming growth factors β1, β3, and platelet-derived growth factor BB (PDGF-BB).2–4

Recently, Yamashita et al5 reported embryonic vascular progenitors capable of differentiating into both endothelial and smooth muscle–like cells in response to vascular endothelial growth factor (VEGF) and PDGF-BB selection, respectively. Presently, there is no evidence for such growth factor–driven differentiation events in adult human subjects. However, there is accumulating evidence from animal studies that smooth muscle cells contributing to vascular disease may originate from bone marrow–derived progenitor cells, with subsequent homing of these cells to experimental atherosclerotic plaque.6–9 These data, in addition to established evidence for circulating adult endothelial progenitor cells (EPCs),6,10,11 suggest the possibility that a distinct smooth muscle progenitor cell (SPC) may also be present in human blood.

Understanding the phenotype of any circulating SPC may have implications for development of novel therapies to modulate homing of these cells to the vessel wall. Intrinsic to this latter understanding may be the identification of specific surface adhesion molecules, such as integrins, which are known to be important in homing of blood-borne progenitor cells to specific sites in vivo.12

In the present study, we tested the hypothesis that outgrowth from human peripheral blood MNC in PDGF BB--
enriched medium would result in SPC differentiation into smooth muscle outgrowth cells (SOCs). Furthermore, because β4 integrins are considered to play a major role in homing of blood-borne progenitor cells and are essential for vascular smooth muscle adhesion, matrix assembly, and cell proliferation, we also hypothesized that the integrin α5β1 (the most highly abundant β1 integrin in proliferating smooth muscle cells in vivo) would be important in SOC adhesion.

Methods

Study Subjects
We used 6 blood samples from 5 healthy human volunteer donors (3 male and 2 female, age 26 to 38 years) according to a protocol previously approved by the institutional review board. Fresh blood was collected by venipuncture and anticoagulated in citrate phosphate dextrose solution (Baxter).

Buffy Coat Preparation and Vascular Progenitor Cell Culture
Human mononuclear cells (MNCs) were initially isolated from peripheral blood buffy coat in Histopaque-1077 followed by washing in MCDB 131 supplemented with hydrocortisone, antibiotics, and 10 ng/mL VEGF. Mononuclear cells were then resuspended in EGM-2 medium and placed on 3 wells of a 6-well plate coated with collagen type I (Becton Dickinson). At 4 weeks, subconfluent cell colonies were passaged and cells were subsequently cultured in either EGM-2 to maintain endothelial cell phenotype or EGM-2 supplemented with PDGF BB (50 ng/mL, R&D Systems) to facilitate smooth muscle cell differentiation. Human vascular smooth muscle cells (hVSMCs) were obtained from Clonetics, and human fibroblasts (hFBs) were obtained from ATCC. In separate experiments, CD34+ve mononuclear cells (90% purity) were selected using immunomagnetic beads and the MACS technique (Miltenyi Biotech), and these cells were similarly differentiated on collagen type I matrix, as described above.

Evaluation of Smooth Muscle Outgrowth Cell Phenotype
Morphological appearance and indirect immunofluorescence were used to define smooth muscle cell phenotype. Primary antibodies were used against CD34 (Immunotech IM 1869), α smooth muscle actin (αSMA), smooth muscle myosin heavy chain (MHC), and calponin (all from Dako Corp). In each immunofluorescence experiment, an isotype-matched IgG control was also used. Binding of primary antibodies to progenitor cells was detected with Alexa-Fluor 488–conjugated anti mouse IgG (Eugene, Oreg). Antibodies to human vWF (Dako Corp, Carpenteria, Calif), VE-Cadherin (Santa Cruz Biotechnology, Santa Cruz, Calif), and CD31 (Sigma Co, St Louis, Mo) were used to label EOC, as previously described. These markers allowed definition of cells as smooth muscle or endothelial lineage.

Western Blot Analysis
Western blotting was performed to identify vascular smooth muscle cell–specific cytoskeletal protein, VEGF receptor, and Tie-2 receptor expression in SOCs. Briefly, cells were homogenized in lysis buffer containing 50 mmol/L Tris HCl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/mL PMSF, and 1 μg/mL aprotinin. The lysate had protein content determined by Bradford assay, and equal amounts of protein were denatured by boiling, reduced in 1 mmol/L DTT, followed by electrophoresis in 12% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose and immunoblotted using monoclonal antibodies to αSMA, human smooth muscle MHC, human calponin, and Flk1 (Santa Cruz Biotechnology) and polyclonal antibodies to Fli1 (R&D Systems, Minneapolis, Minn) and Tie-2 receptor (Santa Cruz Biotechnology, Santa Cruz, Calif) at dilutions of 1:500. Secondary anti-mouse, anti-rabbit, and anti-goat antibodies conjugated to horseradish peroxidase (Calbiochem, San Diego, Calif) at a 1:1000 dilution were used for detection using chemoluminescence (Supersignal, Pierce) and x-ray film exposure (Kodak). hVSMCs and hFBs were used as positive and negative control cells for smooth muscle–specific markers.

FACS Analysis
FACS was performed to identify both cell-surface and intracellular antigens in MNC, SOC, and EOC. Primary antibodies to αSMA, CD31, and integrin α5β1 were used with secondary detection using an FITC-conjugated antibody in each case. Isotype-matched IgG antibodies were used as a control, and the fluorescent intensity of stained cells was gated according to established methods.

Outgrowth Cell Integrin α5β1 Expression and Adhesion Assay
Integrin α5β1 expression on MNC, EOC, and SOC was quantitated using FACS analysis. Integrin α5β1 in EOC and SOC was also analyzed by Western blotting using cell lysates electrophoresed on a 4% to 20% gradient SDS-PAGE. Equal amounts of protein were transferred to nitrocellulose, and α and β subunits were immunodetected using a primary antibody to the human integrin α5β1 (10 μg/mL, Chemicon, Temecula, Calif) and a secondary anti-mouse HRP conjugate (1:500), as described above. Equal loading of protein was confirmed by use of α-tubulin antibody.

To confirm the adhesive function of surface α5β1, integrin expression on each outgrowth cell type, adhesion assays on human fibronectin (10 μg/mL, Sigma) were performed. Both EOC and SOC at a density of 1.5 × 10^5 cells/well on a 6-well culture plate were allowed to adhere in basal medium (EBM-2) with 0.1% BSA in the presence or absence of a primary antibody to human integrin α5β1 (10 μg/mL) or a mouse IgG control antibody at a similar concentration. Nonadherent cells were then washed off, and adherent cells were lifted with trypsin and subsequently counted with a hemocytometer. Percentage adhesion was calculated by dividing the number of adherent cells by the total number of cells plated per well.

Outgrowth Cell Proliferation Assay
Both SOC and EOC at a similar passage were seeded at a density of 5 × 10^4 per well on a 24-well plate coated with collagen type I and incubated overnight with EGM-2 and 5% FCS. Similar initial seeding density was confirmed 12 hours after plating by use of a cell-tierer MTS assay (Cell-Titer AQ, No. G5421, Promega). This generated a baseline seeding absorbance for both cell types. All cells were then grown arrested for 24 hours in serum-free EBM-2. Cells were released from growth arrest with addition of EGM-2 and 5% FCS, and the cell number in each well was determined by cell tier assay at 2, 4, 6, and 8 days after serum stimulation. The absorbance generated at each time point was expressed as a ratio of the initial seeding absorbance obtained for each progenitor cell type.

Statistics
All data are presented as mean ± SEM. Comparison between groups was made using one-way ANOVA. P < 0.05 was considered statistically significant.

Results

Vascular Progenitor Response to Growth Factor Selection
Approximately 6 to 8 colonies per initial patient MNC sample seeded on collagen type I matrix with EGM-2 culture medium (Figure 1A) grew out over a 3-week period, at which time a mixed population existed of polygonal- and stellate-shaped cells (Figure 1B). These mixed cultures were passaged and split into 2 plates, which were subsequently grown in either EGM-2 with high levels of PDGF BB or EGM-2 alone to encourage smooth muscle cell and endothelial cell...
growth, respectively. The cells maintained in the PDGF BB–enriched medium became predominantly smooth muscle–appearing cells with a “hill and valley morphology” (Figure 1C) within an additional 2-week period. These SOCs grew at a rapid rate, achieving >40 population doublings over a 4-month period from the time of initial colony formation. The endothelial outgrowth cells (EOCs) exhibited a typical cobblestone morphology (Figure 1D) and grew at a slower rate, achieving ≈20 population doublings in the 4 months after colony formation. Similar differentiation was seen when using an initial CD34+/ve mononuclear population to derive outgrowth cells.

Immunophenotyping of Smooth Muscle Outgrowth Cells

To additionally evaluate phenotype, cells were stained using smooth muscle cell–specific antibodies. Subconfluent SOC stained positive for αSMA, smooth muscle MHC, and calponin (Figure 2) on indirect immunofluorescence, whereas the starting MNC population and EOC stained negatively for all smooth muscle cell markers (Figure 2). Similarly, hVSMCs in culture stained positively, whereas hFBs stained negatively for all smooth muscle–specific markers (Figure 2). EOCs were confirmed positive for endothelial markers such as CD31, vWF, and VE-Cadherin, whereas SOC stained universally negative for all endothelial markers (Figure 3). In each case, the isotype-matched IgG control antibody stained negatively. To confirm the presence of smooth muscle–specific proteins in SOC but not in MNC or EOC, cell lysates from each cell type were run on SDS-PAGE, immunoblotted, and confirmed to have αSMA, smooth muscle MHC, and calponin protein at appropriate molecular weights (Figure 4). Positive hVSMC and negative hFB controls also showed appropriate presence and absence of immunoreactivity for smooth muscle–specific markers (Figure 4).
To quantify the intensity of staining and the percentage of positive cells expressing αSMA and CD31 in each population (MNC, SOC, and EOC), intracellular (αSMA) and cell surface (CD31) antigens were determined by FACS. The smooth muscle–specific marker (αSMA) was detected in 0% of both MNC and EOC populations, whereas 93% of SOC were positive for this marker at high intensity (Figure 5). Similarly, CD31 was detected in 0% of SOC but in 96% and 99% of MNC and EOC, with a much higher intensity of CD31 staining in the EOC compared with the MNC population (Figure 5).

Hematopoietic origin of SOC was confirmed by positive immunofluorescence staining for CD34 (Figures 6A and 6B). Furthermore, SOC lysates showed significant levels of both VEGF receptors (Flt1 and Flk1) (Figure 6C) on Western blotting, consistent with what has previously been described for endothelial outgrowth cells, whereas SOC lysates were negatively immunoreactive for the Tie-2 receptor compared with EOC (Figure 6C).

Integrin α5β1 Expression, Matrix Adhesion, and Cell Proliferation
Cell-surface integrin α5β1 expression on MNC, EOC, and SOC was quantitated by FACS. SOC showed increased α5β1 intensity and increased numbers of cells staining positive for this integrin compared with EOC and MNC (Figure 7). These data were confirmed by Western blotting of cell lysates, with SOC showing much higher levels of α5 and β1 integrin subunit proteins compared with EOC (Figure 7). To test the functional significance of increased integrin α5β1 expression on SOC compared with EOC, a fibronectin adhesion assay was performed. SOC showed an 8-fold greater adherence to fibronectin compared with EOC (P<0.001), and this effect could be significantly inhibited (P<0.01) using an α5β1 antibody, whereas similar concentrations of isotype-matched mouse IgG had no such effect (Figure 7). Moreover, SOC, when released from growth arrest with serum, had a significantly (4- to 5-fold, P<0.001) increased rate of proliferation compared with EOC of similar passage and seeding density (Figure 8).
Discussion

We report here for the first time the ex vivo outgrowth culture of SOC from putative SPC in human blood. We provide several lines of evidence to demonstrate that hematopoietic mononuclear cells do indeed differentiate in culture into smooth muscle cells. First, SOC grown out from mononuclear cells showed classic smooth muscle morphology and immunophenotype but were CD34 positive, a surface marker known to be absent from adult human smooth muscle cells.22–24 Second, no smooth muscle cell–specific markers were detected in freshly isolated mononuclear cells either by FACS, Western blotting, or immunofluorescence staining. Third, mononuclear cells cultured in the presence of VEGF rather than PDGF expanded into endothelial monolayers that were negative for smooth muscle cell–specific markers by FACS, Western blotting, and immunofluorescence. Fourth, expanded SOC showed a capacity for extended growth in culture (>40 population doublings) in contrast to adult human smooth muscle cells, which become senescent after \( \approx 10 \) population doublings.25,26 Together, these data make it extremely unlikely that the SOC observed in this study resulted from contaminating adult smooth muscle cells.

PDGF BB promoted adult smooth muscle cell differentiation and expansion from progenitor colonies of mixed morphological appearance in this study, whereas VEGF did not. It has previously been shown that PDGF BB is implicated in embryonic smooth muscle cell differentiation,5 and the present study would support a potential role for PDGF BB in differentiation of smooth muscle cells from putative progenitors in circulating blood. Indeed, whereas PDGF BB expression is tightly regulated in vivo,27 it is known to be released from platelets28 and is upregulated at sites of endothelial perturbation and vascular injury.29 Because these sites of PDGF BB expression are precisely where vascular smooth muscle cell proliferation occurs, it is possible to speculate that interaction between PDGF BB and circulating SPC could occur at the blood-vessel wall interface.

SOCs in this study expressed both VEGF 1 and 2 receptors but not Tie-2 receptor, consistent with an angioblastic lineage distinct from EOC that has previously been described as Tie-2 receptor positive.3,9 This nonendothelial phenotype of SOC is supported by a morphological and protein expression phenotype of these cells, which was different from EOCs grown from the same MNC pool, and by a lack of CD31, VE cadherin, vWF, and Tie-2 receptor labeling in these cells.

Integrin \( \alpha_5\beta_1 \) expression and adhesion to fibronectin were markedly increased in SOC compared with EOC in this study, the latter functional effect being significantly inhibited by \( \alpha_5\beta_1 \) integrin antibody. These data suggest that SOC rather than EOC might preferentially attach to fibronectin extracellular matrix. This integrin profile could potentially allow differentiated circulating SPC to attach to sites in vivo, where fibronectin or other \( \alpha_5\beta_1 \) adhesive matrices, such as fibrin,30 are exposed to flowing blood. Because these conditions frequently exist after endothelial perturbation or plaque rupture, it is possible that fibrin clot or exposed subendothelial fibronectin in the vessel wall could serve as the soil in which circulating SPCs attach and proliferate. The proliferative capacity of SOC is supported by much higher rates of in vitro cell growth seen in SOC compared with EOC in this study. Together, these data support a paradigm for circulating SPC with the potential for differentiation, homing, and proliferation at sites rich in extracellular matrix proteins such as fibronectin.

Several in vivo animal studies have suggested that bone marrow–derived smooth muscle cells contribute to transplant arteriopathy and neointima formation after vascular injury and hyperlipidemia.6–9 The present study, by demonstrating smooth muscle cell outgrowth from putative SPC in blood, extends this theoretical framework to human subjects. These findings may have implications for understanding what cells constitute the vasculature in adults, opening new possibilities for diagnosis and therapy of vasoproliferative disease. For instance, monitoring of these cells in blood may enable assessment of atherosclerosis progression, whereas targeting of surface integrins on these cells may inhibit homing to vascular components such as fibronectin. Finally, ex vivo...
expansion of these cells may have implications for cell, gene, and tissue engineering approaches to vascular disease.

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References
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