Isolation of “Side Population” Progenitor Cells From Healthy Arteries of Adult Mice

Julie Sainz, Ayman Al Haj Zen, Giuseppina Caligiuri, Corinne Demerens, Dominique Urbain, Mathilde Lemitre, Antoine Lafont

Objective—Circulating progenitors and stem cells have been reported to contribute to angiogenesis and arterial repair after injury. In the present study, we investigated whether the arterial wall could host permanently residing progenitor cells under physiological context.

Methods and Results—Using the Hoechst-based flow cytometry method, we identified and isolated progenitor cells termed side population (SP) cells at a prevalence of 6.0±0.8% in the tunica media of adult mouse aortas. Arterial SP cells expressed the ATP-binding cassette transporter subfamily G member 2, frequently present on SP cell surface, and displayed a Sca-1+/c-kit-/Lin-CD34-/flow profile. They did not form myeloid or lymphoid hematopoietic colonies after plating in methylcellulose-based medium. Importantly, cultured SP cells were able to acquire the phenotype of endothelial cells (CD31, VE-cadherin, and von Willebrand factor expression) or of smooth muscle cells (α-smooth muscle actin, calponin, and smooth muscle myosin heavy chain expression), in presence of either vascular endothelial growth factor or transforming growth factor-(TGF)-β1/PDGF-BB, respectively. Moreover, they generated vascular-like branching structures, composed of both VE-cadherin<sup>+</sup> cells and α-smooth muscle actin<sup>+</sup> cells on Matrigel.

Conclusions—In this study, we provide the first evidence to our knowledge that in the adult mice, the normal arterial wall harbors SP cells with vascular progenitor properties. (Arterioscler Thromb Vasc Biol. 2006;26:281-286.)

Key Words: endothelial cells • smooth muscle cells • SP cells • vascular progenitors • vasculogenesis

Vascular growth and remodeling are key events in the adaptation of arteries to physiological and pathological environmental stimuli. However, because of the complexity of these processes, questions remain on the underlying mechanisms. During the past years, several studies suggested that bone marrow (BM) circulating stem and progenitor cells could contribute to angiogenesis in animal experimental models of ischemia by incorporating into newly formed capillaries and differentiating into endothelial cells (ECs).<sup>1–3</sup> Moreover, it has been assumed that BM progenitor cells could play a major role during arterial remodeling by integrating into the injured vessel and differentiating into ECs and/or smooth muscle cells (SMCs).<sup>4–6</sup> In recent work, Hu et al alternatively suggested that Sca-1<sup>+</sup> cells found in adventitia of apolipoprotein E (apoE)<sup>−/−</sup> mice do not originate from the BM.<sup>7</sup>

However, no work to our knowledge reported the presence of constitutively resident progenitor cells in healthy adult arteries. Considering that the vascular wall needs to preserve its integrity and homeostasis during lifetime and to provide appropriate response to injury, the existence of progenitor cells residing permanently in the adult arterial wall seems to be quite likely. These cells could allow physiological renewal and provide local rapid rescue for regeneration after injury by rapidly giving rise to new ECs and SMCs, and hence prevent major complications of vulnerable plaque.

The aim of our study was therefore to determine whether tissue-residing progenitor cells could exist within normal adult mouse arteries. To assess their presence, we used the Hoechst DNA binding dye-based method first described by Goodell et al.<sup>8</sup> which enables stem/progenitor cell identification regardless of their tissue origin, and relies on their specific ability to expel the permeant fluorescent dye Hoechst 33342 out of the cell, thanks to a transmembrane transporter called ATP-binding cassette transporter subfamily G member 2 (ABCG2).<sup>9</sup> The Hoechst negative cell population, corresponding to the tissue progenitor cells, can be visualized by flow cytometry and is termed “side population” (SP), because of its paucity and particular peripheral location on fluorescence-activated cell sorter (FACS) analysis dot plot.

Our results demonstrate that the normal adult vascular wall contains arterial SP cells in the tunica media. These cells display phenotypic and, most importantly, functional progenitor cell properties.

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Methods

Animals
All experimental procedures involving animals were performed on 5- to 8-week-old C57Bl/6 female mice purchased from Charles River France Laboratories (Les Oncins, France), in accordance with protocols that complied with National Institutes of Health guidelines.10

Cell Preparation
Thoracic and abdominal aortas were rinsed by perfusion through the left ventricle with HBSS, 2% fetal calf serum, collected from anesthetized C57Bl/6 mice and dissected under direct magnified vision. Surrounding fat and adventitia were carefully isolated from media and intima. Both fractions—adventitia and intima/media—were minced and digested separately during 4 hours with type II collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey) at 400 U/mL, in D-MEM with high glucose (Invitrogen), 2% fetal calf serum under rotation at 37°C in 5% CO2. No living endothelial cells from the intima were found in the digestate of the intima/media fraction (data not shown). Hence, only medial cells in this fraction were further analyzed. Digestates of medial and adventitial cells were passed through 100-mm nylon filter and resuspended in fresh medium to stop enzymatic reaction.

Flow Cytometry
Adventitial and medial cells were stained with Hoechst 33342 (5 μg/mL) and propidium iodide (2 μg/mL) as described previously,5 in presence or absence of verapamil (100 μmol/L, Sigma), bone marrow SP cells being used as positive controls.

Incubation with fluorescent (fluorescein isothiocyanate) antibodies reactive to Sca-1, Lineage Cocktail (CD3e, Gr-1, Mac-1, TER-119, B220), c-kit, Flk-1 or CD34 (Pharmingen, BD Biosciences) was performed at 4°C. Isotype IgGs were used as controls to assess background fluorescence. Flow cytometry analyses were performed on a BD LSR using CELLQuest software (BD Biosciences). Three independent experiments were performed for each immunostaining.

ABCG2 Immunostaining
FACS-sorted arterial SP and main population (MP) cells underwent cytospin immediately after isolation, were stained with rabbit anti-ABCG2 polyclonal antibody (PC-138; Kamiya Biomedical Company, Seattle, Wash) and HRP-conjugated goat anti-rabbit Ig revealed with liquid diaminobenzidine (DAKOCytomation) and counterstained with hematoxylin. Background staining was reduced by using peroxidase-blocking reagent (DAKOCytomation) and 10% goat serum in phosphate-buffered saline (PBS).

For immunohistochemistry studies, aortas and left carotids from anesthetized mice were removed as described, embedded in paraffin, and sectioned in 5-μm sections. Sections were incubated with 5% normal rabbit serum before incubation with rat anti-ABCG2 monoclonal IgG (clone BXP-53; ALEXIS Biochemicals, Lausen, Switzerland) in blocking solution and biotinylated rabbit anti-rat Ig (DAKOCytomation). Staining was amplified using Vectastain ABC peroxidase kit (Vector Laboratories), revealed by diaminobenzidine. Sections were counterstained with hematoxylin. ABCG2-positive and total cells were counted on 30 sections and averaged.

Cell Differentiation Assays
Arterial SP and MP cells were sorted using a BD FACSVantage and immediately cultured on 96-well flat bottom plates for all differentiation assays. A mean of 25 mice aortas was needed to isolate 50 000 to 70 000 arterial SP cells.

For hematopoietic colony assays, 250 to 1000 arterial SP, MP, and bone marrow cells were cultured in MethoCult M3434 or MethoCult M3630, as outlined by manufacturer (StemCell Technologies, France), and myeloid or lymphoid colony appearance was monitored over 3 weeks.

For vascular cell differentiation assays, 7000 SP or MP arterial cells per well were cultured in either EGM-2 or SmGM-3 medium (CAMBREX Bioscience, France) either deprived of VEGF and TGF-β1, respectively, or supplemented with human recombinant VEGF (10 ng/mL), human recombinant TGF-β1 (50 ng/mL), or human recombinant PDGF-BB (20 ng/mL) (Sigma). Media were replaced with fresh preparations every 2 to 3 days. After 15 days, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Background staining was neutralized by biotin-blocking system (DAKOCytomation) and incubation with 10% serum of appropriate species in PBS. Mouse anti–α-smooth muscle actin (ASMA) (clone 1A4; Oncogene Research Products, Boston, Mass), mouse anti-calponin (clone hCP; Sigma), rat anti-CD31 (clone MEC13.3, rat anti–VE-cadherin (clone 11D4.1) (BD Pharmingen), polyclonal rabbit anti-human von Willebrand factor Ig (DAKOCytomation), mouse anti-smooth muscle myosin heavy chain (clone MHC [G-4]; Santa Cruz Biotechnology Inc), and mouse anti-embryonic smooth muscle myosin heavy chain (clone 3H2; ABCAM) antibodies were used for immunostainings, and revealed by biotinylated rabbit anti-mouse Ig, biotinylated rabbit anti-rat Ig (DAKOCytomation), AF488-conjugated goat anti-rabbit Ig, and AF546 or AF488-conjugated Streptavidin (Molecular Probes). Hoechst 33342 (2 μg/mL) was used for nuclei staining. Each experiment was repeated at least 3 times.

Matrigel Culture and Immunostaining
Seven thousand FACS-sorted arterial SP or MP cells were cultured in EGM-2 endothelial cell medium on undiluted Matrigel basement membrane matrix (Discovery Labware; BD Biosciences) in a single well of 96-well plates. After 3 weeks, whole mount was fixed in 4% paraformaldehyde, air-dried at 4°C before ASMA, and VE-cadherin immunostaining was revealed by AF488-goat anti-mouse Ig and AF546-goat anti-rat Ig (Molecular Probes). Experiments were repeated 4 times.

Results
Identification of Arterial SP Cells in the Adult Aortic Wall
Hoechst staining was performed on cells extracted from murine aortas, including all 3 arterial layers. Flow cytometry results showed the existence of a resident Hoechst-negative SP among total murine aortic cells on the left side of dot plot, clearly distinguishable from the clustered Hoechst-positive MP (Figure 1A, left panel). Addition of verapamil—which is known to inhibit ABC transporter function—completely abolished the SP profile, illustrating the specificity of the staining (Figure 1A, right panel).

After identifying SP cells in the normal arteries, we determined their precise localization inside the arterial wall. After arterial tissue dissection, Hoechst staining was performed on cells isolated either from the media or from the adventitia. Results indicated that living SP cells were found only in the media layer (Figure 1B), and represented 6.0±0.8% of living medial cells and 0.9±0.1% of total (living and dead) medial cells.

Only ABCG2 (among ABC transporters) is associated with Hoechst efflux properties necessary for the SP phenotype.9 Immunocytchemistry experiments performed on arterial SP and MP cell cytospins revealed that all arterial SP cells expressed ABCG2 (Figure 2A). In contrast, no ABCG2 staining was found on arterial MP cell surface (Figure 2A), suggesting that ABCG2 was a reliable surface marker to identify SP cells, which was therefore chosen to confirm SP cell localization in situ by immunostaining on tissue sections from murine healthy carotids and aortas. ABCG2-positive cells were found at a prevalence of 5.7±1.3% in the tunica

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media of aorta, in agreement with the data obtained by FACS, and at a prevalence of 15.1±4.3% in carotid (Figure 2B).

**Arterial SP Cell Phenotyping for Stem/Progenitor Markers**

To further characterize arterial SP and MP cells, expression levels of stem or progenitor cell markers were evaluated at their surface by flow cytometry. On one hand, Sca-1 and CD34 distributions were bimodal (composed of two distinct subpopulations with high and low/negative expression) on SP and MP cells (Figure I, available online at http://atvb.ahajournals.org). The percentage of positive cells as well as the level of marker expression indicated as mean fluorescence intensity (MFI) were analyzed (Table). However, c-kit, Flk-1, and Lineage markers were weakly expressed on both SP and MP cells, displaying a unimodal distribution (Figure I). In this case, only the MFI was reported in the Table. Sca-1 cells were abundant in arterial SP (87.6±4.0%) and significantly more than in MP (72.7±1.4%; P<0.05); however, Sca-1 expression was slightly higher on MP cells (MFI=2210±13.13) than on SP cells (MFI=1244.6±16.41; P<0.001). Lineage markers were also more highly expressed on MP cell surface (MFI=486.97±13.03) than on SP cell surface (MFI=331.51±6.43; P<0.001). CD34⁺ cell proportion was similarly low among SP and MP (8.8±0.6% and 14.0±3.9%, respectively, not significant), but CD34 was a little more expressed on SP cell surface (407.29±38.58) than on MP cells (370.12±3.08; P<0.01). In summary, using a cocktail of specific fluorescent antibodies in combination with Hoechst staining, arterial SP cells can be defined as Sca-1⁻ c-kit⁻ low Lin⁻ cells with low CD34 and Flk-1 expression.

**Arterial SP Cells Do Not Generate Hematopoietic Colonies on Methylcellulose**

Because many organs contain stem cells with hematopoietic capacity, we investigated whether arterial SP cells could generate colonies of myeloid or lymphoid-type hematopoietic cells in methylcellulose cultures. No colony was detected in SP or MP cell cultures up to 3 weeks after plating, contrary to

**FACS Analysis of Stem/Progenitor Markers on Arterial SP and MP Cells**

<table>
<thead>
<tr>
<th></th>
<th>MFI</th>
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<tr>
<td></td>
<td>SP</td>
<td>MP</td>
</tr>
<tr>
<td>SCA-1</td>
<td>1244.62±16.41</td>
<td>2210.10±13.13</td>
</tr>
<tr>
<td>CD34</td>
<td>407.29±38.58</td>
<td>370.12±3.08</td>
</tr>
<tr>
<td>C-KIT</td>
<td>38.77±1.60</td>
<td>42.95±1.72</td>
</tr>
<tr>
<td>FLK-1</td>
<td>32.89±0.32</td>
<td>35.76±1.39</td>
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<tr>
<td>LIN</td>
<td>331.51±6.43</td>
<td>486.97±13.03</td>
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</tbody>
</table>

Mean fluorescence intensity (MFI), mean percentage of positive cells (%), ±SEM, (n=3). Student t test.

NS indicates nonsignificant.

*P<0.05; ‡P<0.01; #P<0.001.
bone marrow cell cultures, indicating that SP cells from adult aorta could not differentiate toward neither the myeloid lineage (Figure II, available online at http://atvb.ahajournals.org) nor the lymphoid lineage (data not shown).

**Arterial SP Cells Can Be Committed to Vascular Differentiation**

To evaluate SP cell expression of vascular markers, arterial SP cell cytospins were performed immediately after FACS sorting and immunostained with antibodies against EC and SMC markers. At the basal state, arterial SP cells were all negative for the EC markers CD31, VE-cadherin (CD144), and von Willebrand factor. Regarding SMC markers, all arterial SP cells were negative for calponin and smooth muscle myosin heavy chain (SM-MHC) expression, and only 5% of SP cells were positive for α-smooth muscle actin (ASMA), whereas 95% were positive for embryonic smooth muscle myosin heavy chain (SMemb).

When cultured for 15 days in media deprived of VEGF or TGF-β1/PDGF-BB, SP cells maintained their undifferentiated phenotype as illustrated by their negative staining to assessed EC and SMC markers (Figure 3A and 3B histograms). In contrast, in the presence of VEGF, arterial SP cells became round-shaped, resembling ECs, and part of the cells acquired CD31, VE-cadherin and von Willebrand factor expression (Figure 3A), whereas SP cells cultured with TGF-β1 or PDGF-BB adopted a rather elongated phenotype, similar to that of SMCs, and part of the cells acquired ASMA, calponin, and SM-MHC expression and showed decreased SMemb expression (Figure 3B). Notably, treatment with TGF-β1 induced ASMA and calponin expression in a higher proportion of cultured SP cells, in comparison with treatment with PDGF-BB (Figure 3B).

Similar experiments were performed to study MP cells. In absence of VEGF, MP cells displayed negative staining to CD31, VE-cadherin and von Willebrand factor. However, even without TGF-β1 or PDGF-BB, ≈40% of MP cells expressed ASMA, calponin, and SM-MHC in cultures in contrast to SP cells. After addition of VEGF, MP cells remained negative for CD31, VE-cadherin, and von Willebrand factor staining, whereas addition of TGF-β1 or PDGF-BB induced ≈20% increase in expression of tested SMC markers (data not shown).

**Arterial SP Cells Form Vascular-Like Structures on Matrigel**

To explore the vasculogenic capacities of SP cells, sorted SP or MP cells were cultured on Matrigel matrix scaffold. Interestingly, arterial SP cells created complex vascular-like structures, whereas arterial MP cells did not give rise to any structure (Figure 4A). Within 5 days, SP cells formed spheroids. Cellular structures grew from nascent spheroids, invading the surrounding matrigel by day 12, and branched, forming a vast arborescent structure by day 17 (Figure IIIA, available online at http://atvb.ahajournals.org). Higher magnification revealed that SP cell spheroids could fuse. By day 10, after a first set of cells had sprouted out the spheroids, building structures resembling the framework of a vessel, a second set of cells migrated over the pre-existing framework to form vascular-like structures, invading underlying matrigel or occasionally connecting spheroids together (Figure IIIB). Whole-mount immunofluorescence double-staining documented that the structures consisted of VE-cadherin+ cells and ASMA+ cells (Figure 4B). In summary, arterial SP cells showed vasculogenic potential in vitro by forming complex outgrowing arborescent structures made of both ECs and SMCs.

**Discussion**

Because most organs harbor resident stem cells as a mean to preserve their physiological integrity,13–15 we hypothesized that the arterial wall could similarly contain resident progenitor cells at the basal state, which could participate in
physiological tissue renewal and play a role during vascular growth and remodeling. The present study provides the first evidence that healthy arteries host progenitor cells in adult mouse, termed “arterial SP cells,” which represent 6.0% of medial cells. Because it had been reported that adult tissues harbor stem cells with hematopoietic potential, we investigated whether arterial SP cells could generate hematopoietic colonies. Arterial SP cells displayed no differentiation toward the myeloid or the lymphoid lineage, in contrast with SPs from other tissues. This particularity could originate from the peculiar connections established between the vascular and the hematopoietic system in the embryo. During murine development, the aorta-gonad-mesonephros (AGM) region is the primary site of hematopoiesis. At embryonic day 8.5, hematopoietic cell clusters appear in the floor of dorsal aorta and give rise to hematopoietic stem cells that migrate to fetal liver. Once liver colonization has occurred, AGM hematoietic cell clusters appear in the floor of dorsal aorta and migrate to the liver. Once liver colonization has occurred, AGM hematoietic cell clusters appear in the floor of dorsal aorta and migrate to the liver. Once liver colonization has occurred, AGM hematoietic cell clusters appear in the floor of dorsal aorta and migrate to the liver.

Regarding stem/progenitor cell marker expression, arterial SP cells could be defined as Sca-1$^+$ c-kit$^{lo}$ Lin$^-$ CD34$^{lo}$ and Flk-1$^{lo}$. This profile differs from that of BM SP cells, where c-kit is frequently detected, but is in concord with that reported for skeletal muscle SP cells, which are mainly Sca-1$^+$ (>80%), seldom express c-kit and CD34, and are negative for Lineage cocktail markers. Hence, these results coincide with the general assumption that SP cells from various tissues could share a common set of markers with BM SP cells but also adopt phenotypic characteristics that vary according to the tissue they reside in.

The ABC transporter ABCG2, which confers the SP phenotype by expelling the Hoechst dye, was present on all arterial SP cell surface but none of MP cells, and was therefore used to identify and localize SP cells inside the vascular wall. ABCG2 immunostaining of arterial sections confirmed flow cytometry results showing that arterial SP cells resided in the tunica media. Several studies previously emphasized that the media layer is not a homogeneous SMC population. For instance, calcifying vascular cells (CVC) isolated from bovine aortic media display multilineage potential in vitro. Moreover other investigators reported the existence of peculiar SMC subpopulations, some of them consisting of dedifferentiated, highly proliferative, and migratory cells. Interestingly, we found that arterial SP cells did not express SMC or EC markers at the basal state but expressed the embryonic form of smooth muscle myosin heavy chain, which can notably be found in fetal SMCs. However, part of these cells were able to differentiate into both vascular cell types after appropriate stimulation in vitro, thereby illustrating their plasticity.

Arterial SP cells concealed another property making them clearly distinguishable from other media cells. In contrast with MP cells, arterial SP cells displayed vasculogenic potential, as illustrated by their exclusive ability to give rise to vast arborescent vascular-like structures in vitro. Moreover other investigators reported the existence of peculiar SMC subpopulations, some of them consisting of dedifferentiated, highly proliferative, and migratory cells. In our study, arterial SP cells formed vast arborescent vascular-like structures on Matrigel, which did not resemble classical capillary-like networks formed by ECs or endothelial progenitor cells (EPCs). SP cells first formed spheroids from which branching structures emerged that, unlike networks generated by EPCs and ECs, consisted not only of VE-cadherin$^+$ ECs but also of ASMA$^+$ SMCs. Interestingly, Yamashita et al had previously identified a common vascular precursor cell, which formed tubular structures arising from spheroids in vitro and differentiated into both ECs and SMCs. These structures strikingly resembled those generated by SP cells. However, the vascular progenitor described by Yamashita et al was derived from Flk1$^+$ embryonic stem cells, whereas the present study provides evidence of a progenitor cell population with vasculogenic potential in the adult.

In conclusion, the present work demonstrates the existence of resident SP cells, with phenotypic and functional progenitor cell properties, in the normal adult arterial wall. Their vascular plasticity suggests these cells could participate in arterial homeostasis and remodeling. Moreover, considering their capacity to form vascular-like structures in vitro, these cells could play a major role in vasculogenesis and collateral growth. Arterial SP cells could indeed provide rapid local response to pathophysiological stimuli, BM cells being mobilized in case of more severe arterial trauma. In this perspective, further investigations are warranted to determine
whether arterial SP cells could be used both as a complementary source of progenitor cells in vasculogenesis treatments and as a novel therapeutic target in vascular disease.

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References

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FIGURE LEGENDS

**Figure I: FACS profiling of arterial SP and MP cells.** Stem and progenitor cell marker expression was assessed on SP and MP cell surface by flow cytometry analyses. Histograms showing fluorescence intensity obtained for each marker. Black line indicates cells labeled with isotype control antibody. Filled region identifies cells labeled with antibody for the marker indicated above. Sca-1 and CD34 markers presented a bimodal distribution, whereas c-kit, Flk-1 and Lin markers displayed a unimodal staining pattern.

**Figure II: Hematopoietic colony assay for arterial SP cells.** Microphotographs of arterial SP and MP cells cultured in methylcellulose-based MethoCult M3434 medium for three weeks, bone marrow (BM) cells serving as positive control. Colonies appeared in BM-corresponding wells (top), whereas no hematopoietic colony was detected in SP and MP wells. Scale bars, 500 μm.

**Figure III: Arterial SP cells generate vascular-like structures in vitro.** (A). Kinetic of arterial SP cell culture on Matrigel (d=days). Scale bars, 500 μm. (B), Higher magnification images from the kinetic. By day 5, arterial SP cell spheroids marked by black and white asterisks (*) were capable of fusing together (1 and 2). Cells sprouted from these spheroids (3) and formed vascular-like structures connecting spheroids together (arrowhead) or embedding into Matrigel to ramify (4). Scale bars, 100 μm.
Figure I
Figure III

A.

1d  5d  12d  17d

B.

1.  2.  3.  4.

[Images with annotations]