Adventitial Stem Cells in Vein Grafts Display Multilineage Potential That Contributes to Neointimal Formation

Yikuan Chen,* Mei Mei Wong,* Paola Campagnolo, Russell Simpson, Bernhard Winkler, Andriani Margariti, Yanhua Hu, Qingbo Xu

Objective—This study was designed to carry out the characterization of stem cells within the adventitia and to elucidate their functional role in the pathogenesis of vein graft atherosclerosis.

Approach and Results—A mouse vein graft model was used to investigate the functional role of adventitial stem/progenitor cells on atherosclerosis. The adventitia of vein grafts underwent significant remodeling during early stages of vessel grafting and displayed markedly heterogeneous cell compositions. Immunofluorescence staining indicated a significant number of stem cell antigen-1–positive cells that were closely located to vasa vasorum. In vitro clonogenic assays demonstrated 1% to 11% of growing rates from adventitial cell cultures, most of which could be differentiated into smooth muscle cells (SMCs). These stem cell antigen-1–positive cells also displayed a potential to differentiate into adipogenic, osteogenic, or chondrogenic lineages in vitro. In light of the proatherogenic roles of SMCs in atherosclerosis, we focused on the functional roles of progenitor-SMC differentiation, in which we subsequently demonstrated that it was driven by direct interaction of the integrin/collagen IV axis. The ex vivo bioreactor system revealed the migratory capacity of stem cell antigen-1–positive progenitor cells into the vessel wall in response to SDF-1. Stem cell antigen-1–positive cells that were applied to the outer layer of vein grafts showed enhanced atherosclerosis in apolipoprotein E–deficient mice, which contributed to ≥30% of neointimal SMCs

Conclusions—We demonstrate that during pathological conditions in vein grafting, the adventitia harbors stem/progenitor cells that can actively participate in the pathogenesis of vascular disease via differentiation into SMCs. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: adventitial tissue ▪ atherosclerosis ▪ cell movement ▪ smooth muscle cells ▪ stem cells

Autologous vein grafts remain the only surgical alternative for many types of vascular reconstruction, but the patency rate is still limited because of neointima formation and atherosclerosis within the grafted vessels.2 Neointimal hyperplasia in vein grafts seems to facilitate the acceleration of subsequent vein graft atherosclerosis,2 but the mechanisms of neointimal formation in vein grafts remain unclear. We demonstrated that the earliest cellular event in response to the graft is cell death, either via apoptosis or necrosis,3 followed by inflammation and cell proliferation within the grafts.4 Nevertheless, the hallmark of vein graft pathology includes marked alterations in the adventitial layer of the vascular wall, as indicated by increased cell infiltration and hyperplasia.5 Indeed, cell densities and neovascularization were found to be significantly increased in the adventitia of vein grafts 2 weeks after surgery.6 Although it is known that adventitia plays a part in the neointimal formation after vessel injury,6 the functional roles of defined cell populations within the specialized compartment in the pathogenesis of vein graft atherosclerosis remain clarified.

The adventitia is composed of a vast network of connective tissue and cell populations that include collagen fibers, vasa vasorum, nerve endings, a few quiescent resident inflammatory cells, fibroblasts, and other cells.7 The complex network of cellular and molecular players that serve as building blocks within the vascular adventitia is required to act as a biological processing center for the retrieval, integration, storage, and release of key regulators for functional maintenance of the vessel wall.8–11 More recently, accumulating evidence implicated the presence of stem/progenitor cells within the adventitia of the vessels.12–14 Interestingly, these resident progenitor cells have been demonstrated to differentiate into a variety of cell types, including those of the vascular lineage, in response to specific culture conditions in vitro.15 Although seminal studies implicate the...
importance of adventitial stem/progenitor cells during physiological conditions, the functional roles of adventitial stem/progenitor cells in pathological conditions as seen in vein grafts are not known, especially in their potential contribution toward arteriosclerosis and neointimal hyperplasia. Furthermore, previous work has mostly focused on the putative roles of cellular players that were derived from either the intima or media. In the present study, we hypothesize that stem/progenitor cells in the adventitia of vein grafts can participate in neointimal formation. Thus, we first aimed to investigate the phenotypic and functional characterization of the adventitial-derived stem/progenitor cells from vein grafts. Subsequently, we dissected the mechanisms that mediate their contribution toward the pathogenesis of vein graft arteriosclerosis and confirmed the results in vivo using a vein graft mouse model.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Resident Stem/Progenitor Cells Are Present in the Adventitia of Vein Graft
As previously described by a substantial body of work, the early events after the establishment of a vein graft involve extensive cell loss. This is rapidly followed by an increase in inflammation and uncontrolled proliferation of the intima. Consistently, we observed marked changes in cellular composition within the adventitia of vein grafts in a time-dependent manner (Figure 1A–1F). Indeed, we saw an expedition in the increment of cell numbers from 3 days after surgery onward (Figure 1C–1F) after an initial loss of cells (Figure 1A and 1B). Furthermore, various morphological changes were observed during the duration of vein grafting, including the formation of cell clusters surrounded by microvessels (Figure 1G and 1H; indicated by black arrows) and the presence of populations with large cellular nucleus (Figure 1D and 1E; indicated by white arrows). Importantly, the number of stem cell antigen (Sca)-1-positive cells were significantly increased during the development of vein graft arteriosclerosis (Figure 1K).

Previous work published by our group and others had identified a population of progenitor cells that reside within the adventitia under physiological conditions. In light of the complexity and vast cellular compositions that were seen in our vein graft mouse model, which ultimately result in neointimal hyperplasia, we aimed to identify the presence of progenitor cells within the pathological vessel and investigated their persistence and contribution toward the process. Interestingly, we found that vein grafts at 4 weeks after implantation harbored a small but significant population of cells that express progenitor cell markers, such as Sca-1 (Figure 1I and 1J), CD117, and CD34 (Figure 1A, 1B, and 1D in the online-only Data Supplement); CD34+ cells were found to be <0.2%, and no obvious SSEA-1+ cells were identified (Figure 1D in the online-only Data Supplement). Approximately 12.6% of total cells within the adventitia were positive for macrophage marker, Mac-1 (Figure 1C and 1D in the online-only Data Supplement).

It was intriguing to find that the Sca-1+ cells were localized in close proximity to CD31+ cells lining the vasa vasorum (Figure 1I and 1J), thus postulating a putative role of vasa vasorum in sustaining the survival of Sca-1+ cells. Furthermore, we also detected the presence of smooth muscle cells (SMCs; α-smooth muscle actin–positive) that were found to envelope CD31+ cells that lined the vasa vasorum (Figure 1E in the online-only Data Supplement; indicated by white arrow). Taken together, it is plausible that the progenitor cells that we have identified to be localized within the adventitia of vein grafts may contribute to the pathology of arteriosclerosis, potentially via their differentiation into SMCs.

Clonogenicity and Differentiation of Adventitial Sca-1+ Cells In Vitro
For further characterization of the various cell populations, vein graft explants were seeded in vitro and maintained in stem cell medium. Morphology of the explant-derived cells...
revealed a heterogeneous population that included fibroblast-like, adipocyte-like structured, round-shaped mononuclear cells (Figure IIA–IID in the online-only Data Supplement). Consistently, gene expression analysis confirmed the expression of progenitor cell markers, such as Sca-1, CD34, and CD117 (Figure IIE in the online-only Data Supplement). To clarify the origin of adventitial stem cells, chimeric mouse models were created and vein grafts were performed. The data shown in Figure III in the online-only Data Supplement indicate that adventitial stem cells are derived from the vessel wall, but not bone marrow cells. We next aimed to test their clonogenic capacity by conducting a limiting dilution assay. As a result, we found that ≈5% to 11% of cells developed a primary colony, and ≈30% of those colonies could be expanded further (Figure 2A). The morphology of cells appeared to be markedly variable among the different colonies, thus suggesting that several subpopulations of progenitors are present in the heterogeneous cultures (Figure 2B). Interestingly, although we found that the capacity of differentiation into the endothelial lineage was restricted to few clones (Figure 2C), we observed that all clones consistently showed the ability to differentiate into SMCs (Figure 2D and 2E). In addition to their clonogenicity, the adventitia-derived cells possessed multipotent differentiation capacities and were able to give rise to adipocytes, osteoblasts, and chondrocytes when cultured under specific differentiating conditions (Figure IV in the online-only Data Supplement). Altogether, the results indicate that cells derived from the adventitia are both clonogenic and multipotent.

**Differentiation of Adventitial Sca-1⁺ Cells Into SMCs Is Dependent on Integrin/Collagen IV Interactions**

In light of the data that we have obtained thus far, it is tempting to postulate that adventitial-derived progenitor cells (Sca-1⁺) can contribute to neointima formation after vein grafting, potentially via differentiation into SMCs. To confirm this, we isolated Sca-1⁺ cells from primary cultures using magnetic beads and subjected the cells to SMC differentiation in the presence of collagen IV. Using real-time polymerase chain reaction, we found that collagen IV significantly induced gene expression levels of a panel of SMC markers (SM22α, calponin, α-smooth muscle myosin heavy chain (SMMhC), and α-SMA) were strongly expressed by the majority of clones during differentiation at the protein level (Figure 3A). Concomitantly, the differentiation of progenitor cells caused a decrease in the expression levels of progenitor cell markers, such as Sca-1, CD34, and CD117 (Figure 3D). Taken together, the data showed that Sca-1⁺ progenitor cells derived from the adventitia can differentiate into mature SMC in vitro and, therefore, are likely to participate in the process of neointima formation after vein grafting.
Next, we aimed to elucidate the mechanisms that are involved in SMC differentiation of the progenitor cells. Figure 4A showed that progenitor-SMC differentiation resulted in a marked increase in the expression of several integrins, namely α4, α5, and β1. Consistently, focal adhesion kinase was also found to be activated (Figure 4B), thereby supporting the involvement of integrins in the differentiation process. To confirm the direct role of integrins in inducing SMC differentiation, the progenitor cells were pretreated with an RGD peptide G4391 for 30 minutes to antagonize integrin function before differentiation; RGD peptide S3771 and dimethyl sulfoxide (DMSO) were also used as controls. Data from Western blot showed a marked inhibition of calponin as a result of treatment of RGD peptide G4391, thus confirming the interaction of integrin/collagen IV in regulating progenitor cell differentiation into SMCs.

Adventitial Progenitor Cells Migrate Into Decellularized Vessel Wall in Response to SDF-1

Recently, we have established an ex vivo bioreactor model in which thoracic aorta from mice was decellularized by an overnight treatment with SDS before perfusion with a circulation of culture medium. As a result, translucent acellular vessel scaffolds were generated, whereby fluorescent staining of the vessels with 4',6-diamidino-2-phenylindole (blue) was undetectable, thus indicating the absence of any cell nuclei (Figure 5A).

In this study, we aimed to evaluate the migratory capacities of adventitial Sca-1+ progenitor cells into the acellular scaffold and their subsequent in situ differentiation into SMCs. In consideration that SDF-1 has been established for its role as a potent chemoattractant and is abundantly secreted by key cellular players (ie, macrophages) during atherosclerosis, we wondered whether the chemokine could induce the migration of progenitor cells into decellularized vessels. To address this question, Sca-1+ progenitor cells were applied to the external side of the vessel and allowed to migrate in response to exogenous SDF-1 that was added into the circulating medium. Normal culture medium in the absence of SDF-1 was used as a control. Although we found that a small number of progenitor cells could actively migrate into the scaffold wall in the absence of exogenous stimuli (Figure 5B), the addition of SDF-1 significantly enhanced the number of migrating cells in the decellularized vessels (Figure 5C and 5D). The chemotactic response toward SDF-1 may not be surprising because the

**Figure 3.** Stem cell antigen (Sca)-1–positive cells can differentiate into smooth muscle cells (SMCs). The isolated Sca-1+ cells were cultured in the presence of collagen IV for 6 days. The potential of SMC differentiation was verified by real-time reverse transcriptase polymerase chain reaction (RT-PCR; A), immunofluorescence staining (B; calponin–fluorescein isothiocyanate [FITC] and SM22α–FITC, both green), and Western blot (C). An inhibition of progenitor gene expression was observed using real-time RT-PCR (D). Data are means±SEM (n=3); *P<0.05, **P<0.01, ***P<0.005. α-SMA indicates smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; and SMMhC, smooth muscle myosin heavy chain.

**Figure 4.** The role of integrin/collagen IV interaction in smooth muscle cell (SMC) differentiation. Adventitial stem cell antigen (Sca)-1+–positive cells were cultured in collagen IV–coated dishes for 6 days. Integrins and focal adhesion kinase (FAK) protein expression after differentiation were examined using Western blot analysis (A and B). Adventitial Sca-1+–positive cells were pretreated with RGD peptide G4391 (0.5 mmol/L) for 30 minutes at 37°C before SMC differentiation for 6 days. RGD peptide S3771 (0.5 mmol/L) or dimethyl sulfoxide (DMSO) was also used as control. Cells were harvested for protein expression analysis with Western blotting (C). GADPH was used as a loading control.
Role in neointima formation in vivo. We observed the formation of neointimal lesions that were integrated a dose-dependent upregulation of Sca-1+ progenitor cell migration into decellularized vessel walls expressing marked levels of the SMC marker, SM22α (Figure 5A). In contrast, the expression levels of the endothelial cell marker, CD31, were not detectable (Figure 5F).

To verify the role of SDF-1/CXCR4 axis in progenitor cell chemotaxis, we performed in vitro scratch assays as well as transwell experiments. As expected, the scratch assay indicated a dose-dependent upregulation of Sca-1+ progenitor cell chemotaxis in response to SDF-1 (Figure VB and VC in the online-only Data Supplement). Furthermore, a significant increase in the number of cells that have migrated to the underside of a 0.8-µm pore size Transwell in response to SDF-1 was demonstrated (Figure VD and VE in the online-only Data Supplement), thus confirming the role of SDF-1/CXCR4 axis in progenitor cell chemotaxis. Taken together, these results indicate the capacity of Sca-1+ adventitial cells to migrate through the vessel wall for subsequent differentiation into SMCs in situ. This phenomenon implicates their potential role in neointima formation in vivo.

Adventitial Sca-1+ Cells Enhance Atherosclerosis of Vein Grafts

Although hematoxylin and eosin–stained sections from freshly harvested vena cava of apolipoprotein E–deficient mice showed an integrated endothelium and 2 layers of cellular composition between the intima and media (Figure 6A), we observed the formation of neointimal lesions that were composed of >20 layers of cells in vena cava that was grafted into the carotid artery, most of which consisted of foam cells (Figure 6B; indicated by black arrows). To confirm the role of adventitial Sca-1+ progenitor cells on lesion formation in vivo, the cells were applied to the external side of vein grafts before isografting into apolipoprotein E–deficient mice. Vein grafts in the absence of Sca-1+ progenitor cell seeding were used as a control. To demonstrate the fundamental contribution of the vessel wall, vein graft segments were also irradiated before isografting to prevent cell proliferation. In the controls, atherosclerotic lesions were found to be markedly reduced, showing detachment of neointimal lesions from the rest of the vessels (Figure 6C). In contrast, the seeding of Sca-1+ progenitor cells significantly restored atherosclerotic lesion thickness, as shown in Figure 6D and 6E. These results highly suggest that adventitial stem/progenitor cells that enveloped the outer layer of the grafts could enhance atherosclerosis formation by direct migration from the adventitia into the intima.

To clarify the contribution of adventitial tissues and bone marrows to neointimal formation, adventitial tissue of normal vein from wild-type mouse was carefully removed and replaced by the adventitia derived from SM22-LacZ mouse, which was grafted into wild-type animals. In addition, adventitial tissue of vein from SM22-LacZ mouse was replaced by the adventitia derived from wild-type mouse, which was grafted into wild-type mice. Vein grafts were harvested 4 weeks postoperatively, sectioned, and stained with X-gal and nucleus. Data shown in Figure VI in the online-only Data Supplement indicate that adventitial stem cells could contribute to ≈30% of SMCs in neointimal lesions of the grafts. To explore the possible contribution of adventitial stem cells to other cell types within neointimal lesions, chimeric mouse models were established by bone marrow transfer as described previously. We observed that bone marrow–derived cells mostly contribute to accumulation of inflammatory cells (ie, macrophages.

Figure 5. Ex vivo adventitial stem cell antigen (Sca)-1+–positive cell migration into decellularized vessels. Adventitial Sca-1+ cells were seeded in the outer layer of a decellularized mouse thoracic aorta in a bioreactor system, either in the presence or absence of 100 ng/mL SDF-1 in the circulating medium. After 7 days, the vessels were harvested and stained either with 4',6-diamidino-2-phenylindole (DAPI) to detect cell nuclei (A-D) or with CD31–fluorescein isothiocyanate and SM22α–PE (E and F, respectively). Graph in D represents means±SEM from 3 independent experiments. Three vessels were used in each experiment; *P<0.05.

Figure 6. Adventitial stem cell antigen (Sca)-1+–positive cells enhanced neointimal lesions. Vein segments were either untreated (A and B) or irradiated (C and D) before being isografted into carotid arteries of apolipoprotein E–deficient mice. Adventitial Sca-1+ cells (10⁶ cells) were seeded onto the adventitial side to envelop the vein grafts. Grafted tissue fragments were harvested 4 weeks after surgery and stained with hematoxylin and eosin (H&E); freshly harvested vena cava (A), untreated vein graft (B), irradiated vein graft (C), and irradiated vein graft plus Sca-1+ cell transfer (D). Black squares represent the neointima, and the areas of neointimal lesions in vein grafts were quantified (E) as described in Methods section. Graph in E represents means±SEM, 8 animals per group; *P<0.05.
and T cells) and are very rare in the vessel wall of vein grafts (Figure VII in the online-only Data Supplement). These data provide the basic information of the contributions of adventitial stem cells to neointimal formation.

Discussion

In this study, our vessel graft models of wild-type and apolipoprotein E–deficient mice revealed that the adventitia of pathological vein grafts harbors a significant population of progenitor/stem cells that display active involvement in the development of the disease. Furthermore, we provided new findings that demonstrate the existence of stem cell niche–like structure within the adventitia of vein grafts, some of which were located close to the vasa vasorum and other cell components. These stem/progenitor cells possessed clonogenicity and were able to differentiate into SMCs, endothelial cells, and other mesenchymal lineages. It was, however, noteworthy that most clones, if not all, had the capacity to differentiate into SMCs. Furthermore, the mechanism of SMC differentiation was found to be dependent on collagen/integrin interactions. Interestingly, adventitial stem/progenitor cells displayed chemotactic properties ex vivo, in which they actively migrated from the adventitial side into the vessel wall, thus enhancing vein graft atherosclerosis in apolipoprotein E–deficient mice. Therefore, these findings support the notion that during pathological conditions such as in vein grafting, the adventitia may harbor stem/progenitor cells that can actively participate in neointimal formation by direct differentiation into SMCs.

The identification of colocated stem/progenitor cells with the vasa vasorum within the adventitia of vein graft emerged as one of the key findings in this study. Although the progenitor cells are likely to reside within these specialized niches for survival, potentially via the uptake of growth factors and nutrients that are provided by the vasa vasorum, the mechanisms of progenitor cell release and mobilization from their respective niches for deployment into specific sites within the vessel remain unclear. Furthermore, to date, there remains a lack of direct evidence that elucidates stem cell mobilization from the adventitia. More recently, accumulating data indicate the roles of proinflammatory cytokines and chemokines (ie, tumor necrosis factor-α, interleukin-1, transforming growth factor-β, interleukin-8, and SDF-1) in promoting the perpetuation of adventitial inflammation, some of which could potentially play a role in stem cell mobilization. The present study showed that inflammatory cells, such as macrophages, were mainly located in the adventitia of vein graft, thus postulating a potential role of cytokines and chemokines in direct or indirect mobilization of progenitor cells within the adventitia. Interestingly, several studies have shown that SDF-1 and granulocyte colony-stimulating factor can enhance the mobilization of progenitors in circulation. Indeed, we subsequently demonstrated that the chemokine, SDF-1, was able to drive progenitor cell migration into decellularized vessels of ex vivo bioreactor systems. Although the adventitial progenitor cells responded chemotactically toward exogenous SDF-1, other molecules, such as fibroblast-specific protein 1 and monocyte chemoattractant protein-1, may also appear as promising candidates owing to the following evidence: (1) fibroblast-specific protein 1 secreted by bone marrow–derived inflammatory cells can chemoattract and induce SMC proliferation, and (2) blocking of monocyte chemoattractant protein-1 in vein grafts resulted in a significant reduction in neointimal lesions as a result of inhibition of cell migration. Besides serving as a reservoir of progenitor cells for mobilization in response to appropriate cues, it is noteworthy that vasa vasorum may also act as a means of transport for mobilized progenitor cells to migrate into the intima, where they subsequently proliferate or differentiate into SMCs in response to microenvironmental cues.

Previously, a study using a rat arterial injury model showed that exogenously modified and injected carotid adventitial fibroblasts, but not resident fibroblasts, can migrate from the adventitia toward the luminal side in response to SDF. Although elevated stretch stress as a result of vein grafting led to either apoptosis or necrosis of a majority of endothelial cells and SMCs within the vessel wall, it was also shown that ~60% of SMCs in atherosclerotic lesions of vein grafts were derived from the vessel wall. In the present study, we found that as a result of irradiation, the cells in vein grafts appeared to have lost their ability to migrate and proliferate. Furthermore, the vein graft adventitia–derived Sca-1+ progenitor cells were able to migrate into the intima of vein grafts, thereby enhancing the development of lesions. These data might give an alternative explanation as to why the SMCs within neointimal lesions display a specific migratory and proliferative phenotype. These may reflect different populations or stages of differentiation in the contribution of adventitial progenitor cells to neointimal formation.

Recently, the origins of SMCs found in neointimal lesions have attracted much interest. Traditionally, it is believed that mature SMCs in the media can alternate from a contractile to a synthetic phenotype in response to vessel injury. These SMCs can then migrate into the intima where they subsequently form lesions. However, a recent report indicated that medial-derived mature SMCs were neither capable of migrating nor proliferating, as indicated by in vitro aorta explant cultures and in vivo vessel injury models. Instead, SMCs displaying a synthetic phenotype were mostly, if not all, derived from stem cells present in the vessel wall. Although our study cannot exclude the migration of medial SMC into neointimal lesions, our data support the notion that adventitial stem/progenitor cells actively contribute to SMC accumulation within the neointima of vein grafts. These findings, together with reports from several other groups, may significantly develop our understanding of the potential effects of resident stem cells on vascular pathophysiology, indicating a possible mechanism for stem cells in the remodeling of vein grafts. Together, these findings have some implications for clinical application in the future. For instance, a current strategy for treatment of restenosis is to inhibit SMC proliferation, but the outcome of the clinical end point for the patients was not satisfactory. With the knowledge of stem cell contribution to neointimal formation, a new strategy to direct stem cell differentiation could be considered, for example, enhancing stem cell differentiation toward endothelial lineage to repair the damaged vessels, which may lead to significant improvement for clinic therapy. Although data that address the mechanisms of adventitia stem cell differentiation into SMCs remain scarce, recent
evidence using different progenitor cells indicates the involvement of various signal transductions and signaling pathways in driving the differentiation process. The most established mechanism by which SMC differentiation is regulated is via the binding of transcription factors, SRF and myocardin, to the CArG element of promoter regions of SMC genes, thus leading to the initiation of gene transcription. Previous data from our laboratory demonstrated that several signal transducers, including 

In the present study, we confirmed that the interaction of integrins α4/β5 and β1 with matrix protein domains of collagen IV is crucial for adventitial stem cell differentiation as identified by blocking peptides for integrin receptors. As our ex vivo data on adventitial stem cell migration into decellularized vessel wall indicated an abundance of cells that expressed smooth muscle markers, it is plausible that the infiltrated progenitor cells were driven toward SMC differentiation as a result of direct contact with matrix proteins within the vessel wall. This finding implicates that the directionality and lineage fate of the adventitial progenitor cells could be determined by their active interaction with environmental matrix proteins.

In summary, the present study provided evidence that stem/progenitor cells predominantly reside within close proximity to the vasa vasorum during pathological conditions of vein grafts. Furthermore, we demonstrated that adventitial progenitor cells can migrate across the vessel wall in response to SDF-1 for subsequent differentiation into SMCs, a process mediated by matrix protein/integrin interactions. Altogether, our findings postulate an active contribution of the progenitor/stem cells in the pathogenesis of atherosclerosis in vein grafts, thereby providing more understanding and clarity of disease processes within the vessel wall.

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Disclosures
None.

References

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Recent findings support the presence of vascular stem cells that may contribute to arteriosclerosis. In the present study, we found that stem cells are increasing in the adventitia of vein grafts during neointimal formation. Cloning of the stem cells revealed a heterogeneous population of the diseased adventitial cells, which displayed a potential to differentiate into vascular cells and adipogenic, osteogenic, or chondrogenic lineages in vitro. We uncover a novel direct contribution of adventitia stem cells to lesion development in ex vivo and in vivo models. Thus, stem cells may serve as a putative target for novel therapeutic approaches in vascular diseases. Detailed elucidation of the mechanism involved in stem cell differentiation into either endothelial or smooth muscle cells would enable us to design new drugs that could direct the cell differentiation fate to prevent thrombosis and restenosis of injured vessels.
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Adventitial Stem Cells in Vein Grafts Display Multi-lineage Potential and Contribute to Neointimal Formation

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Materials and Methods

Mice and Vein Graft Procedure
All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Adult male C57Bl/6 mice were purchased from Charles River (Margate, Kent, UK). Transgenic SM-LacZ mice expressing β-gal under the control of the smooth muscle-specific protein SM22 promoter have been described 1. The ROSA26 mouse (The Jackson Laboratory, Bar Harbor, ME) is a β-gal transgenic mouse produced by random retroviral LacZ gene insertion in embryonic stem cells; there are three transcription start sites, involving unknown housekeeping gene promoters 2. ROSA26 mice express β-gal activity in adult tissues. Beta-gal activity of cells from both mice is mainly localized in the nucleus. All transgenic mice are of strain C57BL/6. Three genotypes of LacZ -/-, +/- and +/+ mice were identified using The Jackson Laboratory's PCR protocol (primers:5’-ATC CTC TGC ATG GTC AGG TC-3’ and 5’-CGT GGC CTG ATT CAT TCC-3’). The procedure used for vein grafts was similar to that described previously 3. Briefly, 3-month-old mice were anesthetized using a combination of Hypnorm (25 mg/kg; Veta Pharma, UK) and Hypnovel (25 mg/kg; Roche) administered intraperitoneally. The right common carotid artery was mobilized free from the bifurcation at the distal end toward the proximal, cut in the middle, and a cuff placed over the end. The cuff was made from an autoclavable nylon tube 0.63 mm in diameter outside and 0.5 mm inside (Portex Ltd., Hythe-Kent, UK). The artery was turned inside out over the cuff and ligated. The vena cava vein was harvested from an isogenic donor and grafted between the two ends of the carotid artery by sleeving the ends of the vein over the artery cuff and ligating them together with an 8-0 suture. Vigorous pulsation in the vein conduit confirmed successful engraftment. Some vessel segments were irradiated (2,000 Rads). For cell transfer, Sca-1+ cells (10^5 in 50 µl) were mixed with 1% Matrigel at 4C, applied to the adventitial side to envelope the graft.

Adventitial Cell Culture and Sca-1+ Cell Isolation
The procedure used was similar to that we have described previously 4. The vein graft samples were removed from 2 week postoperatively mice. Under a dissection microscope, graft tissues were carefully harvested and prepared by removing surrounding collective tissues. The graft tissues were minced (about 0.5 mm) and explanted onto a culture flask (Falcon). The flask was incubated in a 5% CO2 incubator at 37°C for 3 hours. Then the flask was turned up, and stem cell medium (American Type Culture Collection, Rockville, Massachusetts, USA)
containing leukemia inhibitory factor (10 ng/ml), 2-mercaptoethanol (0.1 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml) was added and incubated for 6–7 days. Medium was changed every 2 days.

Sca-1+ cells were isolated from primary cultured cells upon reaching 75% confluency using a microbead kit according to the manufacturer’s instructions. Briefly, the cells were detached using trypsin and washed with PBS containing 0.5% BSA, before incubating with anti–Sca-1 immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell suspension was applied over a column equipped with a magnetic cell sorting system (MACS). After washing, Sca-1+ cells were collected. For higher purity of Sca-1+ cells, the same process was repeated using a second column. Purity of isolated Sca-1+ cells was evaluated by immunostaining. Isolated cells were cultured in stem cell medium in a slide bottle and incubated at 37°C for 3 days. After trypsin-EDTA treatment, the cells were seeded in a culture flask (Falcon) and incubated for further 3 days. These cells were used for in vitro and in vivo studies.

Clonogenic Assay
In order to obtain single cell cloning, primary cultured vein graft cells were trypsinized, counted and plated in a density of 100 cells in each gelatin-coated 10cm plate. Each experiment included 5 plates and was repeated 5 times. Clones were observed and counted after 10 days of growth. Cells that formed a clone were collected using 6mm cloning disks soaked in trypsin and then plated in 24 well plates. Clones were progressively split at 80% confluence and expanded for further applications. For each passage the number of clones that expanded successfully was recorded.

EC and SMC Differentiation
The culture-expanded clonal cells or isolated Sca-1+ cells were seeded in a collagen IV-coated 8 well chamber slides for 6 days to induce differentiation into SMC. The cells in Collagen IV-coated plates were also cultured in the presence of 50 ng/ml vascular endothelial growth factor (VEGF; Clonetics Inc) for 10 days to induce endothelial differentiation. After differentiation, cells were fixed with 4% paraformaldehyde and subjected to gene or protein expression analysis for endothelial markers (CD31 and CD144) or for SMC markers (Calponin, SMMhc, α-SMA and SM22α), using either real time or RT-PCR, immunofluorescence staining or Western blotting.

Immunofluorescence Staining
For 5-µm-thick frozen section preparation, vein grafts were harvested and immediately frozen in liquid nitrogen. The procedure used for immunofluorescent staining in the present study was similar to that described previously. Briefly, cell smears and frozen sections were labeled with rat antibodies against mouse Sca-1, CD117, CD34, CD133 (Abcam Ltd., Cambridge, United Kingdom), SSEA-1 (Chemicon Europe Ltd., Hampshire, United Kingdom), Mac-1 (CD11b/18), CD3 (BD Biosciences Pharmingen, San Diego, California, USA), and α-SMA (Sigma-Aldrich, St. Louis, Missouri, USA), or a rabbit anti-CD31 antibody (Abcam Ltd., Cambridge, United Kingdom), and visualized with swine anti-rabbit Ig-Cy3 or rabbit anti-rat Ig conjugated with FITC (Dako Cytomation). Semi-quantitative evaluation was performed at ×400 magnification. The number of positive stained cells in the vein graft wall was counted and expressed as the percentage of total nuclei.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
The procedure used for RT-PCR was similar to that we have described elsewhere. Total RNA was prepared with absolutely RT-PCR Miniprep Kit (Qiagen). Data of the sequence of primers
Western Blotting
Preparation of extracts from cultured cells for Western blotting was performed as described previously and modified. Cells were harvested in lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 2 mM EDTA) containing protease inhibitors for the whole cell lysate. Forty µg of protein was separated by SDS-PAGE on a 4%-12% Trisglycine gel (Invitrogen, Carlsbad, CA, USA) and then transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience Gmbh, Germany) and subjected to standard western blot analysis. Nitrocellulose membranes incubated with antibodies followed by an IgG secondary antibody. To confirm equal loading, membranes were stained with antibody against actin or GAPDH (Santa Cruz Biotechnology).

Decellularized Vessel Preparations
The mice were sacrificed by cervical dislocation and fixed in a supine position. The middle line incision was made and the lung was lateralized to expose the thoracic part of descending aorta. Perivascular tissue was removed by forceps and intercostal arteries were ligated with bipolar electrocoagulator (SN 54.131; Martin). Then distal end of descending aorta was cut and washed with saline solution via puncture at ascending aorta. The descending aorta was stored at 4°C in phosphate-buffered saline. The vessel was placed in 0.075% sodium dodecyle sulfate (SDS) (Sigma, St. Louis, USA) solution on a shaker for overnight (15-18 hours). Then the vessels were washed with 5 ml of PBS 5 times for a period of 20 minutes each on a shaker and then stored at 4°C in PBS.

Decellularized Vessel Applied to Bio-reactor Circulation Ex Vivo
Using a dissecting microscope (Zeiss, KL1500 LCD, Germany) with 5- to 10-fold amplification, a cuff (0.65 mm in diameter and 1 mm in length) made of autoclavable nylon tube (Portex, London, UK) was passed through the two ends of decellularized vessel and fixed by ligation twice with an 8-0 silk suture. Then, the decellularized vessel was connected to a Bio-reactor circulation (an ex vivo circulation system which driven by a pump). To study the adventitial progenitor cells migrating across the vessel wall ex vivo, Sca-1+ cells (10^6 in 100 µl) were mixed with Matrigel (5 mg/ml; Becton Dickinson Labware, Bedford, Massachusetts, USA) at 4°C, and applied to the adventitia enveloping the decellularized vessel for 15 minutes static at room temperature. Decellularized vessel was immersed in the culture medium and the ex vivo circulation system was placed in a 5% CO2 incubator at 37°C for 5 days. The circulation system with low shear (10 dynes/cm²) used culture medium for smooth muscle cell differentiation containing stromal cell derived factor-1 (20 ng/ml). Medium was changed every 2 days.

FACS Analysis
The cells were harvested by incubating with trypsin, and centrifuged briefly to obtain a cell pellet. The harvested cells were then washed with ice-cold PBS once, and centrifuged for 5 minutes at 1000 rpm and room temperature. The cell pellet was then re-suspended with 100 µl ice-cold PBS. Cells were labelled with antibodies against Sca-1, c-kit, CXCR4, CD34, and Flk-1 (BD) and analysed with a FACS scan flow cytometer (Becton Dickinson Immunocyto metry Systems, Mountain View, CA, USA).

Migration Assays
Sca-1+ (2x10^5 per well) progenitor cells were seeded in 12-well plates and allowed to attach onto the surface overnight. A scratch was made in the centre of the wells using a 1ml pipette tip before the cells were washed twice with PBS. Exogenous SDF-1 protein was added dose-
dependently (1-100 ng/ml) in each well and the cells were allowed to migrate towards the ‘scratched’ area overnight. Data was expressed as the mean number of migrated cells in 5 random fields of view (at 20x).

Migration assays were performed in transwell inserts with 8.0 µm pore membrane filters (Becton Dickinson Labware, USA). Sea-1⁺ progenitor cells were harvested by treatment with trypsin-EDTA and subsequently loaded onto the upper chamber at 2x10⁴ cells/ml, while exogenous SDF-1 (100 ng/ml) was added to the bottom chamber. Serum-free media was used as a control. After an overnight incubation, the upper side of the filters was carefully washed with PBS and remaining non-migrating cells were removed with a cotton tip applicator. Cells on the underside of the membrane were fixed with 3% PFA for 10 mins before staining with 1% crystal violet solution (diluted with dH2O) at room temperature for 15 minutes. Data was expressed as the mean number of migrated cells in 5 random fields of view (at 20x).

Bone-marrow transplantation. Donor mice were killed and femurs and tibias were removed aseptically. Marrow cavities were flushed with Ca⁺⁺, Mg⁺⁺-free Hanks' Balanced Salt Solution (HBSS) (GIBCO-BRL, Grand Island, NY) using a 25-gauge needle attached to a syringe. Single cell suspensions were prepared by repeat pipetting and the cell preparations passed through a nylon mesh to remove particulate matter. Cells were washed twice in HBSS, counted using a hemocytometer, and resuspended at 3x10⁷ cells/ml before transplantation. Six to eight week old mice received a lethal dose of whole body X-ray irradiation (950 Rads). The irradiated recipients received 1x10⁷ bone-marrow cells in 0.3 ml RPMI1640 by tail vein injection. Vein grafts was performed 4 weeks after bone marrow transfer.

Graft Harvest and Section Preparations
The grafts were harvested in the first, second, third or four post-operative weeks. The mice were anaesthetised and fixed as describe above. The middle line incision of neck was made and the surround tissue was removed to expose the whole segment of the vessel graft and left common carotid artery. Normal saline perfusion was done from the right common carotid artery, after the left common carotid artery was then cut open. The graft was harvested from the bilateral end of the cuff and frozen in liquid nitrogen immediately. For frozen sections, the graft was embedded in optimal cutting temperature aqueous compound (VWR Scientific, Bridgeport, NJ) in a cryostat (Microm HM 560, Walldorf, Germany) at -20°C. The cross sections were collected, from different regions of the graft.

X-gal staining. Mice were anesthetized and perfused with 0.9% NaCl solution and subsequently perfusion fixed with 2% formaldehyde and 0.2% glutaraldehyde (pH 7.2) for 2 and 10 min, respectively. Vein segments were harvested, the samples were fixed with 2% formaldehyde and 0.2% glutaraldehyde at 4°C for 24 h, sections (5-µm) were prepared. The procedure for X-gal staining was similar to that described previously. Briefly, sections were incubated at 37°C for 18 h in PBS supplemented with 1 mg/ml X-Gal (Sigma, St Louis, MO), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂. Sections were rinsed with 3% DMSO in PBS and mounted with the endothelium up on a glass slide (2.6x7.5 cm). Counterstaining was performed using Hoechst 33258 (1 µg/ml). Positive cells were enumerated under the microscope.

Histology and Lesion Measurement
The grafts were fixed with 4% formaldehyde, processed by routine histology and embedded in paraffin. Sections (5 µm) obtained at the centre of the graft were stained with hematoxylin and eosin (HE) for histological evaluation. The procedure for lesional area measurement is similar
to that previously described\textsuperscript{9, 10}. Briefly, the lesion was defined as the region between the lumen and the media. Using a transmission microscope (Zeiss, Jena, Germany) sections were scanned, saved and then overlaid with different lines to trace the lumen and media. The lesion area was determined by subtracting the area of the lumen from the area enclosed by the line inside of the media.

**Statistics**

All experiments were conducted 3-6 times and significant differences were calculated as *p<0.05 compared to control by *t*-test or one-way ANOVA using GraphPad Prism 5.

**References**


Figure I. Characterisation of vein graft adventitial cellular compartment. Immuno-fluorescence staining of adventitial areas from four weeks-old autografts of vein segments. Antibodies against CD117 (FITC), CD34 (FITC), Mac-1 (FITC), CD31 (FITC) and α-SMA (PE) were used (A-C and E). Positive cells were quantified against total cell nuclei (DAPI) (D). Graph in panel D is representative of means ± SEM (n=5)
Figure II. Morphological characterization of cultured adventitial cells. Adventitial cells from 4-week vein grafts were cultured in vitro and showed variable morphologies using hematoxylin and eosin (H&E) staining (A-D). RT-PCR (E) confirmed the presence of cells expressing progenitor markers.
Figure III. Non-bone marrow origin of Sca-1+ cells from vein grafts. Chimeric mice were created by bone marrow transfer. Femurs of ROSA26-LacZ bone marrow was harvested and transplanted to wild-type mice (A and B). The chimeric mice were grafted with vein derived from wildtype animals. The grafts were harvested 4 weeks after surgery, sectioned and stained for β-gal, i.e. panel A as a negative control without X-gal and panel B showing positive staining. Panel C and D: Adventitial cells from 4-week vein grafts of ROSA26-LacZ (A) and chimeric (B) mice were cultured in vitro and passaged for 3 times and then developed with X-gal. Blue color stained cells are positive.
Figure IV. **Multipotency of adventitia-derived progenitor cells.** Adventitia-derived cells were cultured in presence of various differentiation cocktails for specific differentiation into adipogenic, osteogenic or chondrogenic lineages. After 14 to 21 days of differentiation, treated cells showed positive staining for specific antigens indicating bona fide differentiation into adipocytes (FABP4, fatty acid binding protein 4; B), osteoblasts (osteopontin; D) and chondrocytes (collagen II; F). As a negative control undifferentiated cells were stained and showed no positivity for these markers (A, C, E, respectively).
Figure V. The effect of SDF-1 on adventitial progenitor cell migration in vitro. Flow cytometric analysis revealed that in vitro-cultured adventitial Sca-1+ stem cells from 4-week vein graft expressed low levels of the SDF-1 receptor, CXCR4 (A). The progenitor cells indicated increased chemotactic migration in response to exogenous SDF-1, as shown with a scratch assay (B and C; dose dependent manner) and using an 8.0μm transwell assay (D and E). Graphs are means ± SEM (n=3); ***p<0.005.
Figure VI. The percentage of β-gal+ cells in neointimal lesions of vein grafts. Adventitial tissue of normal vein from wildtype mouse was carefully cut away and replaced by adventitia derived from SM22-LacZ mouse, which was grafted into wildtype mice (Left panel). Adventitial tissue of vein from SM22-LacZ mouse was carefully removed and replaced by the adventitia derived from wildtype mouse, which was grafted into wildtype mice (middle panel). Wildtype vein was grafted to wildtype mouse as a control (Right panel). Vein grafts were harvested 4 weeks postoperatively, sectioned and stained with X-gal and nucleus. Note the positive (blue) cells indicated by the arrows. Graphs are means ± SEM (n=5).
Figure VII. Creation of chimeric mice and immunostaining for neointimal cells. Femurs of ROSA26-LacZ bone marrow was harvested and transplanted to wild-type mice (WT; Left group). The chimeric and wildtype mice were grafted with vein derived from wildtype (left group) and ROSA26 (right group). The grafts were harvested 4 weeks after surgery, sectioned and double stained for β-gal and cell markers, i.e. Mac-1 for macrophages, CD3 for T cells, calponin for SMCs. Double positive cells in neointimal lesion were enumerated and expressed as % against total nucleus. (n=5).