Stem Cells and Transplant Arteriosclerosis

Qingbo Xu

Abstract—Stem cells can differentiate into a variety of cells to replace dead cells or to repair damaged tissues. Recent evidence indicates that stem cells are involved in the pathogenesis of transplant arteriosclerosis, an alloimmune initiated vascular stenosis that often results in transplant organ failure. Although the pathogenesis of transplant arteriosclerosis is not yet fully understood, recent developments in stem cell research have suggested novel mechanisms of vascular remodeling in allografts. For example, stem cells derived from the recipient may repair damaged endothelial cells of arteries in transplant organs. Further evidence suggests that stem cells or endothelial progenitor cells may be released from both bone marrow and non–bone marrow tissues. Vascular stem cells appear to replenish cells that died in donor vessels. Concomitantly, stem/progenitor cells may also accumulate in the intima, where they differentiate into smooth muscle cells. However, several issues concerning the contribution of stem cells to the pathogenesis of transplant arteriosclerosis are controversial, eg, whether bone marrow–derived stem cells can differentiate into smooth muscle cells that form neointimal lesions of the vessel wall. This review summarizes recent research on the role of stem cells in transplant arteriosclerosis, discusses the mechanisms of stem cell homing and differentiation into mature endothelial and smooth muscle cells, and highlights the controversial issues in the field. (Circ Res. 2008;102:1011-1024.)

Key Words: stem cells ■ endothelial progenitors ■ smooth muscle progenitors ■ common progenitors ■ arteriosclerosis

Stem cell research is becoming a promising field because of the unique capability of stem cells to differentiate into different types of cells and to regenerate damaged tissues and organs.1 In general, stem cells can be divided into embryonic stem cells and tissue-resident or adult stem cells. It is well known that embryonic stem cells can be obtained from hollow sphere-shaped embryos of 200 to 250 cells, known as blastocysts, which can differentiate into all tissue types.2 Alternatively, adult stem cells are derived from blood, bone marrow, vessel wall, and other tissues that display variable capacities of differentiation. Specifically, the vessel wall- and bone marrow–derived progenitors seem to be crucial in the development of cardiovascular diseases.3,4 Recently, it was demonstrated that bone marrow and the arterial wall contain a number of vascular stem/progenitor cells that have an ability to differentiate into endothelial cells (ECs) and smooth muscle cells (SMCs), respectively.5,6 These cells may directly or indirectly participate in the formation of the vascular lesions, including native atherosclerosis, injury-induced neointimal hyperplasia, vein graft atherosclerosis, and transplant arteriosclerosis.4

Transplant-accelerated arteriosclerosis in the arteries is the major limitation to long-term survival of patients with solid organ transplantation. Transplant arteriosclerosis is character-
ized by diffuse, uniform, concentric narrowing of the artery owing to proliferative, fibrocellular intima. A hallmark of lesions is mononuclear cell infiltration into the vessel wall of grafts at the early stage, followed by neointimal formation, which largely constitutes from SMCs. The etiology of transplant arteriosclerosis is thought to be immune-mediated reactions to donor ECs and SMCs, in which inflammatory cells participate in the progression. Although the pathogenesis of transplant arteriosclerosis remains obscure, the development of organ transplantation animal models and the use of new techniques, eg, stem cell labeling and imaging in vivo, provide new insights into the mechanisms of the disease progression. This review focuses on the role of stem cells in transplant arteriosclerosis and discusses the latest developments associated with stem cell maintenance of endothelial integrity, neointima formation, and stem cell differentiation within the arterial wall.

**Endothelial Damage and Regeneration by Stem Cells**

As mentioned above, endothelial cells in the donor vessels can be damaged by both cellular and humoral components from the recipient. Common early intimal findings include endothelial activation, which is histologically manifested as hypertrophy or a “hobnail” appearance with eosinophilic transformation of the cytoplasm. This correlates with functional activation, including upregulation of major histocompatibility complex class II and adhesion molecules. Endothelial damage can occur via various mechanisms, including direct antibody and complement-mediated or cytotoxic T-cell–mediated apoptosis, the result of which is a loss of barrier function and the influx of clotting proteins (including fibrin), platelets, blood cells, and lipids. Edema, deposition of intercellular matrix, accumulation of lipid, and an increased local turnover of cells are seen in all 3 vessel layers but are especially common within the intima. Taken together, these processes result in disruption of intimal homeostasis and trigger the stem cell repair response, typically seen in the arteries of all types of allografts.

It is a key issue to know how dead ECs are replaced and which cells are responsible for regenerating the endothelium in allografts. According to traditional concept, damaged ECs in allograft vessels can be replaced by cell replication within the graft tissue and are therefore donor-derived. However, this concept is challenged by recent findings that damaged/lost ECs within the allografted vessels are replaced by cells derived from recipients. Several independent groups have reported the contribution of recipient stem cells to endothelial regeneration. For example, conclusive data were obtained using transgenic animals in which expression of LacZ genes was controlled by specific endothelial promoters. This resulted in only the ECs of these mice (TIE2-LacZ) express β-galactosidase (β-gal). Using the animal models for allograft vessels, we performed vessel grafts in two types of transgenic mice expressing β-gal in ECs, including TIE2-LacZ, TIE2-LacZ/apoE−/−, and wild-type mice. We demonstrated that the endothelium on allografts completely disappeared because of apoptosis or necrosis and were replaced by recipient stem cells, of which about one-third of cells were derived from the bone marrows. Furthermore, the number of CD34+ and Flk1+ progenitor cells in blood of apolipoprotein (apo)E-deficient mice were significantly lower than those of wild-type controls, which coincided with diminished β-gal+ ECs on the surface of the grafts in TIE2-LacZ/apoE−/− mice. These findings indicate the contribution of recipient stem cells to regenerate damaged endothelium of the vessel wall (Figure 1). Even microvessel ECs in transplant organs could be regenerated by recipient-derived stem cells, with results supporting the notion that recipient-derived stem cells can repair severely damaged endothelium.

Published data indicate that bone marrow–derived stem cells contribute to the regeneration of the endothelium of allograft vessels, but the percentage of stem cells reported to incorporate into the damaged vessel is variable (Table 1). Using TIE2-LacZ mouse chimeric model, it was shown that approximately 30% ECs within the allograft vessels were derived from bone marrow. In a recent study, Feng et al demonstrated that a large proportion of bone marrow–derived stem cells can directly repair the endothelium. This report also demonstrated that human apoA-I transfer increases the number of circulating endothelial progenitor cells (EPCs), enhances their incorporation into allografts, promotes endothelial regeneration, and attenuates neointima formation in a murine model of transplant arteriosclerosis. Alternatively, Hillebrands et al reported that less than 3% of ECs on allograft vessels were derived from bone marrows in a rat transplant model. This variability between results could be attributable to the use of different techniques, ie, section versus en face analyses (Table 1).

In humans, significant endothelial cell replacement by circulating progenitor cells also occurs in transplant vessels. Simper et al demonstrated that subjects who underwent sex-mismatched cardiac transplantation, displayed significant seeding of recipient ECs (range: 1% to 24%) of all luminal ECs in large-vessel lumens and adventitial microvessels of arteriopathic vessels. No opposite-sex chimeric cells were observed in control sex-matched transplantation scenarios. Similarly, ECs within cardiac allograft in humans were partially replaced by extracardiac progenitor cells, ie, recipient stem cells, which occurs early and could relate to injury during allograft harvest or transplantation. This result was confirmed by a third group showing that endothelial chimerism was common and irrespective of rejections in humans with organ transplantation (Table 1). However, Lagaaj et al reported that endothelial replacement by the recipient in a renal transplantation is associated with vascular rejection. The high degree of endothelial chimerism may have immune implications such as for myocardial rejection or graft arteriosclerosis. These data suggest that a significant number of recipient stem cells are recruited to the lumen of epicardial vessels and adventitial microvessels of coronary artery segments following cardiac transplantation; a process in which transplant arteriosclerosis is associated along with reduction in circulating endothelial precursors.

**Sources of Stem/Progenitor Cells**

During last 5 years, a large number of publications described adult stem cells present in various kinds of tissues and organs (Figure 2). The term “stem cell” and “progenitor” are
often used to indicate the same cell type, because the nature and characterization of stem cells are not well understood. For instance, Sca-1 and c-Kit double-positive cells exhibit stem cell–like properties, differentiating into more than 3 types of mature cells, eg, cardiomyocytes, ECs, and SMCs. However, they are often called “progenitor cells.” In accord, data from embryonic stem cells indicate that ECs and SMCs share the same progenitor. In the present review, the term “stem cell” is used to describe more pluripotent cells, whereas “vascular progenitor cells” refer to the cells that can specifically differentiate into either ECs or SMCs, ie, EPCs and smooth muscle progenitors.

Table 1. Endothelial Cell Origin in Allograft Vessels

<table>
<thead>
<tr>
<th>Species and Allograft</th>
<th>Organ</th>
<th>Method</th>
<th>Recipient</th>
<th>Donor</th>
<th>Bone Marrow</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Mouse</td>
<td>Aorta</td>
<td>TIE2/LacZ</td>
<td>&gt;95%</td>
<td>&lt;5%</td>
<td>~30%</td>
<td>Hu et al&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM chimera</td>
<td>Aorta</td>
<td>GFP</td>
<td>Majority</td>
<td>NA</td>
<td>~20%</td>
<td>Feng et al&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat</td>
<td>Heart</td>
<td>Y-PCR</td>
<td>~100%</td>
<td>0</td>
<td>&lt;5%</td>
<td>Hillebrands et al&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM chimera</td>
<td>Aorta</td>
<td>MHC/HIS52</td>
<td>&gt;95%</td>
<td>NA</td>
<td>~3%</td>
<td>Hillebrands et al&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human</td>
<td>Heart</td>
<td>Y-probe</td>
<td>NA</td>
<td>95%</td>
<td>NA</td>
<td>Hruban et al&lt;sup&gt;17&lt;/sup&gt;</td>
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<tr>
<td>Sex-mismatch</td>
<td>Heart</td>
<td>Y-probe</td>
<td>42%</td>
<td>58%</td>
<td>NA</td>
<td>Quaini et al&lt;sup&gt;95&lt;/sup&gt;</td>
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<tr>
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<td>Heart</td>
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<td>24.3%</td>
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<td>NA</td>
<td>Minami et al&lt;sup&gt;28&lt;/sup&gt;</td>
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<tr>
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<td>Y-probe</td>
<td>1–24%</td>
<td>NA</td>
<td>NA</td>
<td>Simper et al&lt;sup&gt;29&lt;/sup&gt;</td>
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<tr>
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<td>MHC/Y-probe</td>
<td>33–66%</td>
<td>&lt;30%</td>
<td>NA</td>
<td>Lagaaij et al&lt;sup&gt;21&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BM indicates bone marrow; GFP, green fluorescent protein; MHC, major histocompatibility complex; NA, not available; Y-PCR, Y chromosome–specific PCR; Y-probe, Y chromosome–specific probe.
EPCs are a heterogeneous population of cells in circulating blood that express a panel of cell markers, including CD34, FlK-1/KDR/VEGFR2, and CD133. This was initially reported by Asahara and colleagues, who isolated circulating angioblasts from human peripheral blood. They showed that these cells could differentiate into ECs and contribute to neoangiogenesis after tissue ischemia and, consequently, defined this cell population as EPCs. As a consequence, no simple definition for EPCs presently exists, although several groups have set out to define this cell population more accurately. In general, EPCs are defined as cells that express CD34 or, the more immature marker CD133, and an endothelial marker protein such as vascular endothelial growth factor receptor (VEGFR)2. However, a controversial report recently showed that human CD34/AC133/VEGFR-2 cells are not EPCs but distinct, primitive hematopoietic progenitors, indicating that a uniform definition of EPCs is needed to clarify the origins. It could be hypothesized that EPCs originate from numerous sources and from a variety of tissues, which may be responsible for the stem cell pool in the blood displaying the heterogeneity in nature (Figure 2).

To date the bone marrow is the most defined source of circulating EPCs. From here, the EPCs are released in both physiological and pathophysiological conditions, a process which involves several factors/enzymes. They play a part in either cutting the linking between progenitors and matrix net tissues or releasing the progenitors from the tissue. A key factor controlling progenitor mobilization via release of soluble Kit-ligand is matrix metalloproteinase-9. This enzyme enables bone marrow repopulating cells to translocate to a permissive vascular niche favoring their differentiation. Soluble Kit-ligand induces the release of stromal cell–derived factor (SDF)-1 from platelets, enhancing neovascularization through mobilization of CXCR4 progenitors. Pharmacological mobilization of bone marrow stem cells has also been demonstrated following administration of granulocyte colony-stimulating factor. Granulocyte colony-stimulating factor also activates progenitor cell releasing factors such as neutrophile elastases and matrix metalloproteinases, which release the stem cells from the bone marrow into the circulation. Thus, there is no doubt that bone marrow is a unique source of stem cells participating in endothelial repair after alloimmune-induced endothelial loss in grafted vessels. However, it was also reported that a large proportion of EPCs in circulating blood is of non–bone marrow origin. For example, the spleen is an organ particularly rich in EPCs. The adipose tissue also contains stem cells that can differentiate in vitro into ECs. These cells are capable of incorporating into an ischemic leg, can increase capillary density, and improve postnatal neovascularization. In addition, the intestine and liver containing a large number of mobilized tissue-residing progenitor cells have been discovered recently using a parabiosis model (Figure 2). Moreover, it is known that there is an abundant presence of progenitor cells within the adventitia of the arterial walls. To determine the origin of these adventitial cells, chimeric mice that express LacZ transgene only in bone marrow cells were used. No β-gal cells were identified 8 months after the marrow transplantation, implying that these cells are not derived from the bone marrow. Importantly, Hu et al provided direct evidence that non–bone marrow stem cells contribute to endothelial replacement in allograft vessels. Here, the aorta from a BALB/c mouse was allografted into the carotid artery of a chimeric C57BL/6J mouse with bone marrow derived from TIE2-LacZ mice. β-gal activity was seen on the surface of allografts 4 weeks postsurgery. Quantification of the obtained data indicated that more than 70% of the regenerated ECs were derived from non–bone marrow tissues. Interestingly, in a separate study, it was shown that in rat aortic, but not cardiac, allografts, recipient-
derived ECs replaced damaged donor endothelium on the arteries. Even further evidence indicates that the observed contribution of bone marrow–derived ECs in the late stage of transplant atherosclerotic lesions was very low. Therefore, non–bone marrow–derived stem cells may be largely responsible for regeneration of lost ECs in transplant arteriosclerosis.

**Stem Cell Homing to Allograft Vessels**

Early alloimmune response to the ECs of donor arteries results in activation and/or damage to the endothelium that may stimulate release of cytokine and chemokines, forming a chemokine gradient within the vessel wall. Multiple molecules, including families of adhesion molecules and chemokines, provide signals for the dynamic trafficking of stem cells to the surface of allograft vessels. Chemokines are proinflammatory cytokines that function as potent chemottractants for stem cells. They provide signals leading to selective recruitment of the cells at sites of inflammation that may be critical in the process of acute rejection. The chemokines are a large family that can be divided into 4 subfamilies, CXC, CC, C (also called lymphotactin), and CX3C, depending on the position of the first 2 cysteine residues. Each subfamily has relatively specialized chemotaxtractant properties for different cells. Chemokines act on responsive stem cells through G protein–coupled 7-transmembrane receptors. Presently, 5 receptors for CXC chemokines, 11 receptors for CC chemokines, and 1 receptor each for CX3C and lymphotactin have been identified. Using a mouse aortic transplantation model, Sakihama et al show that SDF-1 is a critical molecular target for the progenitor homing. During the course of transplant arteriosclerosis, intragraft SDF-1 expression was upregulated, and the circulating stem cells expressing its counter-receptor CXCR4 increased in the recipients receiving allografts. CXCR4+ stem cells, derived from transplant recipients, migrated into allografts via microvessels in the adventitia and then toward the luminal side. In support of a functional role for these molecules, in vivo neutralization of SDF-1 inhibited stem cell homing. A low-molecular-weight fucan compound has been demonstrated to increase plasma SDF-1 levels and appeared very effective in a rat cardiac allograft model to reduce neointimal lesions. Thus, interaction between SDF-1 and CXCR4 plays a key role in transplant arteriosclerosis development.

Once stem cells are attracted by chemokine to the activated/damaged endothelium, these cells may adhere to the endothelium via highly expressed adhesion molecules in allograft vessels. It was demonstrated that ECs of allografts express all types of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, P-selectin, and E-selectin. Knockout of the ICAM-1 or VCAM-1 gene results in reduced neointimal lesions of allografts, indicating the impact of adhesion molecules, . It could be hypothesized that highly expressed adhesion molecules in allografts may be responsible for stem cell attachment. Supporting this hypothesis is the observation that EPCs expressing the selectin ligand P-selectin glycoprotein ligand-1 have increased adhesion to P-selectin and E-selectin. Meanwhile, small interfering RNA for P-selectin abrogates this response, indicating that P-selectin glycoprotein ligand-1 expression facilitates the recruitment of EPCs and thus enhances their proangiogenic capacity. Furthermore, β2-integrins expressed on the cell surface of EPCs mediate the firm adhesion and transmigration of EPCs to the damaged endothelial monolayer. Thus, interaction between EPC surface molecules and their counter ligands existing in activated/dying ECs of allografts plays a major role in EPC homing.

As described above, alloimmune response to the grafted vessels may severely damage ECs, leading to exposure of subendothelial matrix proteins that activate platelets to adhere to the “naked” areas or to form microthrombi. Interestingly, several groups showed that platelet adhesion not only triggers vascular thrombosis but also represents the critical step for the targeting of EPC homing to sites of endothelial disruption. In vivo, it was demonstrated that CD34+ c-Kit+/Sca-1−/Lin− EPCs directly adhere to platelets after vascular injury in a process that involves platelet P-selectin and GPIb-integrin. Once activated, platelets secrete chemokine SDF-1, which supports primary adhesion of EPCs on the surface of arterial thrombi. These studies identify a central role of platelets for tethering EPCs to the arterial intima, a process of fundamental importance for vascular repair and regenerating lost ECs in allograft vessels.

**Stem Cell Differentiation Into ECs in Allograft Vessels**

Theoretically, adhered stem cells to the surface of allografts with damaged endothelium could differentiate into ECs. However, to date, there are no data concerning stem cell differentiation in allografts in vivo. Subsequently, the present section will discuss data on stem cell differentiation obtained from other models, eg, vascular injury, which may share some similarities in terms of cell differentiation. Obviously, microenvironment of stem cells located in the vessel wall could play a crucial role in determining the fate of the cells. Based on published data, it seems that 2 signals are required for stem cell differentiation: (1) interaction between adhesion molecules/integrin family members and (2) growth factor/shear stress. For example, expression of adhesion molecules by activated ECs of allografts and integrins expressed on the stem cell membrane leads to tethering of stem cells to the surface of the vessel wall and initiates differentiation. The latter, growth factor, could be released by activated ECs and/or platelets aggregated on subendothelial matrix.

The microenvironment of the platelet-rich fibrin clot is most supportive for CD34+ cell differentiation toward EC phenotype, in which VEGF is abundant. Platelets elaborate an array of factors that are involved in wound healing, of which many factors also play a role in the biology of ECs. For instance, the potent growth factor VEGF, which is highly accumulated in procoagulant regions, not only leads to recruitment of EPCs into the damaged areas but also stimulates the differentiation of CD34+ progenitors into ECs. Once CD34+ progenitors attach to the injured vessel wall, they will be subjected to fluid shear stress, which enhances their VEGFR2 expression, proliferation, and tube formation. Data published by Pelosi et al indicate that CD34+
progenitor differentiation into ECs. Additionally, we demonstrated that collagen IV/integrin interaction and VEGF stimulation is essential for Sca-1 progenitors expressing VEGFR2 constitute a functional sub-population that has the capacity to differentiate into ECs. These data provide evidence that may translate to the in vivo situation of stem cell repair to damaged ECs of allograft vessels during development of transplant arteriosclerosis.

As stated above, laminar shear stress created by blood flow stimulates stem cell differentiation. Stem cells attached to the damaged surface of allograft vessels are subjected to the shear stress caused by blood flow, which may directly stimulate cell differentiation. Supporting this notion is the fact that shear treatment of stem cells or EPCs in vitro results in expression of a panel of EC markers, including CD31, ICAM-1, and VE-cadherin. Assays for tube formation in the Matrigel showed that the shear-stressed EPCs form tube-like structures and develop an extensive tubular network significantly faster than the static controls. These findings indicate that EPCs are sensitive to shear stress and that this factor may modulate their maturation. The mechanisms of shear-induced stem/progenitor cell differentiation seem to involve several signal initiators and transducers, as recently identified by Zeng et al (Figure 3). It was shown that histone deacetylase (HDAC) activation is essential in this process. HDACs comprise at least 17 genes, of which HDAC1, HDAC3, and SIRT1 are expressed in human peripheral blood–derived endothelial progenitors, whereas embryonic stem cells express the majority, if not all, of HDAC family genes (L. Zeng, Xu Q, unpublished data, 2008). Three different classes of human HDACs have been defined based on their homology to HDACs found in Saccharomyces cerevisiae RPD3 (class I), HDA1 (class II), and SIR2 (class III). We found that shear stress can rapidly activate the VEGF receptor/Akt/endothelial NO synthase (eNOS) pathway in embryonic stem cell–derived progenitors, in which Akt also induces HDAC3 phosphorylation. One downstream target for HDAC3 is p53, which is upregulated by shear stress. Taken together, shear stress is a positive signal for stem/progenitor cell differentiation into ECs via pathways similar to those used by VEGF (Figure 3).

In adult cells, the endothelial lineage commitment of circulating blood– or bone marrow–derived progenitor cells requires HDAC activity. The inhibition of HDACs decreased the expression of the transcription factor HoxA9 and reduced the number of ECs derived from different progenitor sources. HoxA9 regulates various typical endothelial marker proteins that are also important for the functional activity of ECs. HoxA9 deficiency reduces endothelial lineage commitment and results in severe impairment of neovascularization. The pharmacological inhibition of class I and II HDACs by structurally different pharmacological HDAC inhibitors abrogated HoxA9 expression and the endothelial commitment of progenitor cells. Furthermore, the HDAC inhibitor trichostatin A decreased both eNOS protein and mRNA levels. Although trichostatin A enhanced the activity of the eNOS promoter, it did not alter the rate of eNOS transcription, suggesting that trichostatin A posttranscriptionally targets eNOS mRNA. These data indicate that HDAC-dependent mechanisms contribute to the regulation of eNOS expression, which, in turn, contributes to endothelial differentiation (Figure 3).

Smooth Muscle Heterogeneity in Transplant Arteriosclerosis

The major cellular component of transplant arteriosclerotic lesions is SMCs, which contribute significantly to the development of stenosis. An important feature of smooth muscle biology is the considerable heterogeneity in their phenotypes and origins during development of neointimal lesions in allograft vessels. In the arterial wall, SMCs show a different behavior characterized by an exceedingly low rate of proliferation/turnover, largely nonmigratory, and an extremely low rate of synthesis of extracellular matrix components. More specifically, SMCs in adult animals are highly specialized cells whose major function is the contraction and regulation of blood vessel tone-diameter, blood pressure and blood flow distribution. In pathological conditions, studies suggest that the role of SMCs appears to vary depending on the stage of the disease, playing a maladaptive role in lesion development and progression. In response to endothelial damage of alloimmune response, SMCs of allograft vessels increase their rate of cell turnover and synthetic capacity, contributing significantly to neointimal formation.

It is known that medial SMCs and those within arteriosclerotic lesions differ largely and that a noticeable attempt has been made to examine this phenotypic switching between
normal and disease states.\textsuperscript{80,81} It is believed that before SMCs can migrate from the media into intima, during the formation of arteriosclerosis, a transition in their phenotype is required.\textsuperscript{82} Medial nonproliferating SMCs have a contractile phenotype that enables them to regulate vascular tone. When SMCs proliferate, they acquire a synthetic phenotype. According to Hiltunen et al.,\textsuperscript{83} the proliferative state of the SMC requires profound changes in gene expression and protein synthesis. However, the location, mobilization, and function of SMCs within the vessel wall, in addition to the development of transplant arteriosclerosis, are still poorly understood. Obviously, a major challenge in understanding the (de)differentiation of vascular SMCs is their ability to appear in a wide range of phenotypes at different stages of lesion development. One explanation for smooth muscle heterogeneity is that smooth muscle could undergo dedifferentiation from mature phenotypes to less mature cells, forming arteriosclerotic lesions and appearing at different stages of cell dedifferentiation. Indeed, a large number of publications provide evidence supporting this concept, which has been extensively reviewed.\textsuperscript{82} However, recent data from several groups disagree with the “dedifferentiation concept,” suggesting that SMCs and smooth muscle–like cells within transplant arteriosclerotic lesions may be derived from diverse sources, including circulating blood progenitors, medial stem cells, transdifferentiation of ECs, and adventitial progenitor cells.\textsuperscript{84–86} Derivation from these different sources may be the main reason why SMCs in arteriosclerotic lesions display a diversity of phenotypes, characteristics, and behaviors. Because this is a fundamental issue for understanding the pathogenesis of transplant arteriosclerosis, the sections that follow focus on smooth muscle origins and the mechanisms of SMC differentiation.

**SMC Origins in Transplant Arteriosclerosis**

The traditional view of the formation of neointimal lesions in transplant organs is that SMCs from the media of affected arteries proliferate and migrate into the subendothelial space or intima in response to signals from inflammatory cells and damaged endothelium of the vessel wall.\textsuperscript{87} According to this view, intimal SMCs in transplant arteriosclerotic lesions should originate from the donor vessels. However, recent data obtained from different laboratories demonstrated the recipient origins of SMCs in transplant arteriosclerosis (Table 2).\textsuperscript{19,20,88–93} In rat models, Hillebrands et al.\textsuperscript{19} performed an analysis in sex-mismatched models of aortic and cardiac transplantation, in which severe transplant arteriosclerosis developed after allografting. They demonstrated that all neointimal SMCs in both aortic and cardiac allografts were of recipient origin in renal allografts. Analysis of the reciprocal combinations, however, clearly demonstrated the existence of a persistent population of recipient-type cells.\textsuperscript{94} Additionally, using a mouse model in which SMCs displayed SM22-driven β-gal expression, Hu et al.\textsuperscript{90} have provided conclusive evidence that arteriosclerotic SMCs of vascular allografts also originated from recipients. Consequently, these data all support the recipient origin of SMCs in neointimal lesions in animal models.

In humans, SMC origin in transplant arteriosclerosis of cardiac allografts varies, with a low percentage found to be recipient derived.\textsuperscript{94,95} In contrast to human cardiac allografts, Grimm et al.\textsuperscript{96} showed that 60% to 80% of neointimal SMCs were of recipient origin in renal allografts. Analysis of the reciprocal combinations, however, clearly demonstrated the existence of a persistent population of recipient-type cells.\textsuperscript{94} Alternatively, a report by Atkinson et al.\textsuperscript{97} showed that in sex-mismatched transplants (female to male), no double staining of SMCs/Y chromosome–positive cells could be identified within the neointima, although inflammatory cells marked with Ham-S6 were positive for Y chromosome probe. This study does not support the notion of the recipient origin of SMCs in the neointima of transplant arteriosclerosis.

<table>
<thead>
<tr>
<th>Table 2. Smooth Muscle Cell Origin in Allograft Vessels</th>
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<tbody>
<tr>
<td><strong>Species and Allograft</strong></td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
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<td>BM chimera</td>
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BM indicates bone marrow; GFP, green fluorescent protein; Y-PCR, Y chromosome–specific PCR; Y-probe, Y chromosome–specific probe; NA, not available.
Whether organ-specific differences in this process exist (which may explain the observed differences between the cardiac and renal transplants) remains to be studied. Nevertheless, it is conceivable that SMCs in human transplant arteriosclerosis are derived from both donors and recipients (Table 2).

**Smooth Muscle Progenitors in the Vessel Wall**

As described above, different sources of cells may be responsible for smooth muscle accumulation in transplant arteriosclerosis, in which the stem/progenitor cells in the arterial wall may directly contribute to the process. In fact, early observations by Benditt and Benditt have established a monoclonal theory of SMCs in the lesion, in which smooth muscles displayed a monoclonal origin, ie, derived from a single cell. According to this theory, SMCs in arteriosclerotic lesions could originate from one stem cell that may be present in the arterial wall. It was eventually discovered that the arterial wall indeed contains stem cells that can differentiate into SMCs (for a review, see elsewhere). The previous report from Demer and colleagues identified a subpopulation of vascular cells derived by dilutional cloning of bovine aortic medial cells and showed that they have the potential to differentiate along mesenchymal lineages. For example, chondrogenic potential of these stem cells was evidenced by expression of types II and IX collagen and cytochemical staining for Alcian blue. Alternatively, leiomyogenic potential of these cells was evidenced by the expression of smooth muscle α-actin, calponin, caldesmon, and myosin heavy chain, whereas stromogenic potential of the stem cells was evident by the ability to support growth of colony-forming units of hematopoietic progenitor cells from human CD34 umbilical cord blood cells. These results suggest that the artery wall contains stem cells that have the potential for multiple lineages, similar to mesenchymal stem cells but with a unique differentiation repertoire.

Similarly, Hu et al reported that the adventitia in aortic roots of apoE-deficient mice harbored large numbers of cells expressing stem cell markers, eg, Sca-1 and c-Kit. Isolated stem/progenitor cells are able to differentiate into SMCs in response to platelet-derived growth factor (PDGF)-BB stimulation in vitro. These vascular progenitor cells may differentiate into SMCs, contributing to cell accumulation in arteriosclerotic lesions in vivo. Sainz et al identified and isolated progenitor cells termed “side population cells” at a prevalence of 6.0% in the tunica media of adult mouse arteries. Arterial side population cells express the ATP-binding cassette transporter subfamily G member 2, frequently present on the cell surface, and display a Sca-1<sup>+</sup>/c-Kit<sup>−</sup>/Lin<sup>−</sup>/low CD34<sup>−</sup> profile. Importantly, these cells are able to acquire the phenotype of SMCs in the presence of transforming growth factor (TGF)-β1/PDGF-BB. Thus, the normal arterial wall harbors stem cells with vascular progenitor properties.

In humans, Zengin et al reported the existence of stem cells in a distinct zone of the vascular wall that are capable to differentiate into mature ECs, hematopoietic cells, and macrophages. This zone, localized between smooth muscle and adventitial layer of human adult vascular wall, predominantly contains CD34<sup>−</sup>/CD31<sup>−</sup> cells, which also express VEGFR2 and TIE2. These data suggest the existence of a “vasculogenic zone” in the wall of adult human blood vessels, which may serve as a source for progenitor cells for postnatal vasculogenesis. Concomitantly, a small number of progenitor cells were identified within neointimal lesions and the adventitia with variable expression of CD34, Sca-1, c-Kit, and VEGFR2 markers but no CD133 expression. On average there is a 2- to 3-fold increase in progenitor cell number in the adventitia of atherosclerotic vessels compared with normal arteries, with a significant difference in the frequency of cells expressing VEGFR2. Thus, vascular progenitor cells exist within the arterial wall, and increased numbers of progenitor cells in the adventitia of human atherosclerotic vessels have been identified. Subsequently, these stem/progenitor cells in the vessel wall could be a source of SMCs from the donor vessel during development of transplant arteriosclerosis.

**Smooth Muscle Progenitors in Blood**

Because SMCs in transplant arteriosclerosis of allograft organs are, at least in part, derived from the recipients, it is conceivable that smooth muscle progenitors or stem cells could present in circulating blood. Simper et al demonstrated for the first time that smooth muscle progenitors indeed exist in the blood. They cultured human mononuclear cells, isolated from the peripheral circulation, in a PDGF-BB–enriched growth medium and subsequently identified the presence of smooth muscle progenitors. Furthermore, a recent report confirmed the presence of blood smooth muscle progenitors that are derived from CD14/CD105 double-positive cells. These cells, derived from human blood, proliferate readily and express proinflammatory genes during early culture. After long-term culture, the cells could contract and express characteristic SMC markers, eg, smooth muscle α-actin. The number of CD14/CD105-bearing cells increases significantly in patients with coronary artery disease compared to patients without the disease. These results support the novel concept that smooth muscle progenitors exist in human circulating blood and may contribute to the pathogenesis of vascular diseases.

Smooth muscle progenitors isolated from human blood have also investigated for their integrin profile to provide clues into the homing process of these progenitor cells. Studies by Simper et al and Deb et al showed that these cells have a distinct integrin expression profile compared to endothelial outgrowth cells. Both groups are in agreement and conclude that the β1-integrin is present in greater quantities on smooth muscle progenitor/outgrowth cells than those of endothelial outgrowth cells. The authors highlight the potential importance of integrins in mediating adherence of smooth muscle progenitors to specific extracellular matrix both in vitro and in vivo. In addition, the smooth muscle outgrowth cells also showed a greater adherence to fibronectin, which is known to be adhesive to β1-integrin. Future studies are required to identify the source of smooth muscle progenitors in blood, including bone marrow and non–bone marrow origins.
Bone Marrow–Derived SMCs: A Controversial Issue

Because of the presence of smooth muscle progenitors in blood, it is logically considered that these progenitors may be derived from the bone marrow and that these cells may participate in smooth muscle accumulation in neointimal lesions of allografts. To date, the issue of bone marrow–derived SMCs participating in transplant arteriosclerosis remains controversial (Table 2). Two groups provided data supporting the concept of bone marrow–derived SMCs participating in the neointimal formation. Sata et al102 showed a major contribution (82%) of bone marrow–derived SMCs to the development of transplant arteriosclerosis after cardiac allografting in mice, whereas Shimizu and colleagues88 found a 10.8% involvement. Both studies indicate that SMCs in arteriosclerotic lesions of allografts can derive from bone marrow stem cells. Using in situ hybridization techniques to identify Y chromosomes in human sex-mismatched cardiac allografts, researchers have consistently identified on the order of 3% to 15% recipient-derived intimal SMCs,104 although no direct evidence of bone marrow origins was provided.

Results opposing the contribution of bone marrow–derived SMCs to transplant arteriosclerosis also exist. Using bone marrow chimeric rats, which allow for discrimination between bone marrow– and non–bone marrow–derived cells, Hillebrands et al18 used confocal laser-scanning microscopy along with major histocompatibility complex class I haplotype-specific antibodies, to identify the presence of bone marrow–derived neointimal SMCs in aortic allografts. These experiments revealed that the recipient-derived SMCs are predominantly, if not all, derived from a non–bone marrow source. Similarly, this observation is in line with reports of others20,91 showing a minor contribution of bone marrow–derived SMCs in the development of transplant arteriosclerosis after experimental aortic allografting in mice. Concomitantly, using a mouse model in which Lac Z gene expression is controlled by the smooth muscle specific SM22 promoter, we performed arterial allografts in chimeric mice with SM22-LacZ bone marrow. Interestingly, we were not able to find any SM22-LacZ gene–positive cell in the neointimal lesions of allografts, again suggesting a non–bone marrow origin of SMCs.20 Additionally, this controversial involvement of non–bone marrow– versus bone marrow–derived SMCs in the formation of the lesions also extends to studies of native atherosclerosis and vascular injury-induced restenosis.105–107

What are reasons for such opposing results? There are several possibilities to explain it. Firstly, a possible explanation comes from interpretation of results. For example, data presented from duel labeling for α-actin/β-gal to detect bone marrow–derived SMCs using different imaging resolutions were obtained by different groups. However, it is possible that double-positive cells, initially identified as SMCs, may in fact be SMCs and leukocytes in adjacent regions that were too close to be separately recognized in sections of transplant vessels. Indeed, in an alternate study, sections of graft vessels labeled for α-actin (red) and MAC-1+ macrophages (green), a proportion of double-positive cells (yellow) were identified, even though these cells are separately present in neointimal lesions.108 Additional support also comes from recent articles showing that observation of vessel sections using high-magnification and a higher-resolution imaging technique failed to confirm the bone marrow origin of α-actin+ cells in the lesion.107,109 Secondly, the criteria used to identify SMCs present in the lesions of allograft vessels are different between research groups. What is the definition for SMCs? There is a panel of markers to identify SMCs, including α-actin, SM22, myosin heavy chain, calponin, and smoothelin, but how many markers should be used to determine whether they are SMCs? The conclusions obtained from different laboratories are based on the results using different smooth muscle markers. Finally, stem cell–derived SMCs are different from medial mature SMCs in terms of proteome, although both types of cells express smooth muscle markers, i.e., α-actin, SM22, myosin heavy chain, calponin, and smoothelin.110 Therefore, it is not surprising to see different conclusions by using different techniques and criteria.

The Mechanism of Smooth Muscle Differentiation

SMCs in transplant arteriosclerotic lesions display heterogeneities, different phenotypes from medial cells, and different proteome between mature and stem cell–derived SMCs. Because of this, it would be helpful for us to understand more detailed mechanisms of smooth muscle differentiation from stem cells. In vivo, stem cells or smooth muscle progenitors reside in a special microenvironment called a niche, which could maintain their self-renewal ability. In response to vessel grafts, stem/progenitor cells are mobilized from a variety of tissues,111 and homing to subendothelial space occurs. The stem cell then stays in a specific microenvironment resulting in differentiation.84 Such microenvironmental cues include high concentrations of cytokines or growth factors and extracellular matrix proteins and different mechanical force induced by blood flow, which may determine the fate of stem cell differentiation.4 Under the endothelium of the arterial wall, there are abundant matrix proteins, e.g., collagen, fibronectin, and elastin, to which stem cells attach via the surface integrins. Different types of extracellular matrix proteins may exert different effects on stem cell differentiation, leading to specific cell lineages. In a recent study, we found that collagen IV seems to favor stem cell differentiation toward SMCs.35 Embryonic stem cells seeded on collagen IV–coated plates spontaneously differentiated into SMCs, giving rise to a highly pure population of the cells, which express a number of specific smooth muscle markers.35 It is obvious that the interaction between collagen IV and integrin α1/β1/α4 is essential for stem cell differentiation into SMCs.

After tethering to the collagen IV, stem cells may need an additional signal to initiate the differentiation, in which TGF-β and PDGF-BB play a crucial role. Recent data demonstrated that PDGF-BB and TGF-β promote vascular progenitor cells derived from the adventitia and media of the arterial wall differentiate into SMCs.33,35 The downstream signal-transduction pathways involve focal adhesion kinase, phosphatidylinositol 3-kinase and mitogen-activated protein

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kinase. More recently, Margariti et al. provided the evidence that HDAC7 mediates smooth muscle differentiation, although it also plays a role in endothelial migration and angiogenesis. It was found that HDAC7 undergoes alternative splicing during stem cell differentiation. In mature SMCs, the spliced HDAC7 isoform is mainly expressed, whereas stem cells have unspliced form. On HDAC7 splicing, the short isoform binds to myocyte enhancer factor (MEF2C), a transcription factor responsible for muscle marker protein expression, leading to MEF2C degradation via proteasome and suppression of smooth muscle differentiation. The signal pathways of stem cell differentiation into SMCs have been illustrated in a schematic figure (Figure 4).

Additional information concerning smooth muscle differentiation involving epigenetic modification can be found in a recent review article published in this journal.

A Common Progenitor for Endothelial and SMCs

In the process of normal embryonic development, the majority of ECs are derived from angioblasts (or hemangioblasts) of the aorta–gonad–mesonephros, whereas most vascular SMCs arise from local mesenchyme. On the other hand, studies on adult tissues showed that stem/progenitor cells present in a variety of tissues can differentiate into both ECs and SMCs. Consequently, the question arose whether both ECs and SMCs in aortic vascular lesions are derived from the same progenitor. It seems possible, although direct evidence of such a common progenitor participating the pathogenesis of transplant arteriosclerosis is still lacking. In embryonic stem cells, a common Flk-1+ progenitor can differentiate into ECs and SMCs in response to different growth factors. We have also identified Sca-1+ progenitors derived from embryonic stem cells and the adventitial tissue of adult apoE−/− mice, which have an ability to differentiate into ECs and SMCs when stimulated by VEGF and PDGF, respectively. Similarly, laminar flow induces stem cell differentiation toward EC lineages, whereas it suppresses SMC differentiation. It was also observed that mesenchymal stem cells can differentiate into both ECs and SMCs and that vascular progenitor cells are able to differentiate into contractile-type smooth muscles in the absence of VEGF or into synthetic-type phenotypes in the presence of PDGF-BB, respectively. In addition, immune suppression drug cyclosporine can inhibit both endothelial and smooth muscle progenitor cell growth and thus influences neointimal formation in transplant models.

In human embryonic stem cells, a report demonstrated that a population of vascular progenitors with the ability to differentiate into endothelial-like and smooth muscle-like cells has been identified. Specifically, vascular progenitors isolated from an embryonic body grown in suspension express endothelial marker CD34. When these cells are subsequently cultured in endothelial growth medium supplemented with VEGF, they give rise to endothelial-like cells characterized by a cobblestone cell morphology and expression of various endothelial markers. In contrast, when CD34+ cells are cultured in EGM-2 supplemented with PDGF-BB, they give rise to smooth muscle–like cells, characterized by spindle-shaped morphology, and to SMC marker expression. These findings provide direct evidence that stem cell differentiation to smooth muscle and ECs may be mediated through a common vascular progenitor, which, depending on the local microenvironment, activates specific pathways and directs the differentiation process.

Although the data collected from different sources support the concept that ECs and SMCs can differentiate from a common vascular progenitor, the underlying mechanism involved in the transition is still very limited. Further investigation to elucidate the genetic changes and specific signal pathways involved in the transition of ECs and SMCs from a common progenitor will provide novel approaches to select cell differentiation. This will open exciting opportunities, such as new therapeutic strategies for vascular disease.

Limitations of Animal Models

In the present review, a large proportion of the data cited are derived from the experimental results of animal models. Although the mouse and rat offer an incredibly valuable tool for the study of transplant arteriosclerosis in the laboratory, it is essential for the investigator to be aware of similarities and differences that exist between animal models and human disease. We must bear in mind that results obtained in the experimental animals may not translate directly to humans. Several crucial issues should be addressed when the data from mice are interpreted to transplant arteriosclerosis in humans. First, mice weigh approximately 25 g, some 3000 times less than the average man. As a result, anatomic and physiological conditions vary between the mouse and human.
For instance, human coronaries are located on the epicardium, whereas rodent coronary arteries are largely intramural, in which the endothelial layer lies directly on the internal elastic lamina, and the media consists of only 2 or 3 layers of SMCs. Second, some models of mice, eg, heterotopic heart grafts, do not mimic physiological or pathological situations in humans. Third, in terms of drug usage, immunosuppression is often limited to subclinical levels, or no immunosuppression agent used in some animal models. Suboptimal immunosuppression may result in much greater acute vascular rejection/injury, which differs from patients with organ transplantation. Finally, some reports indicate that the majority of cells in human transplant vessels are from the donor even years after transplantation. In contrast, most of the ECs and SMCs in experimental aortic and heart allografts in animal models can be of recipient origin. This large discrepancy between rodent models and human transplants is summarized in Tables 1 and 2. Consequently, caution must be advised when extrapolating from mouse model data to supposed human equivalents.

Conclusions and Future Perspectives

Alloimmune-induced endothelium damage could be an initiator for the development of transplant arteriosclerosis. Following the endothelial injury, stem cells derived from different sources may participate in endothelial repair and SMC accumulation. In this process, the fate of stem cell differentiation into either ECs or SMCs is a key issue for the progression of transplant arteriosclerosis (Figure 5). Based on the understanding to the novel mechanisms of the pathogenesis of transplant arteriosclerosis, ie, contribution of stem cells, a new therapeutic strategy for the disease could be emerged. Stem cell research is unique in that it focuses on the prospect of regenerative medicine. When translated to the field of vascular disease, the potential therapeutic uses of donor-derived, or patient-derived, stem cells are far reaching and include enhancing endothelial repair of damaged vessels, promoting reendothelialization, and preventing stenosis in vascular allografts. However, much needs to be learned about the mobilization, homing, differentiation processes, and mechanisms of these processes, ultimately aiming to define a complete molecular classification of the exact cell type that repairs damaged vessels and that is responsible for SMC deposition in the intima.

Recently, several independent groups have published excellent works on adult cell reprogramming into stem-like (iPS) cells by introducing 4 genes (Oct4, c-Myc, Sox2, and Klf4) into fibroblasts. These iPS cells can differentiate into different types of cells in vitro and in vivo. In combination with other technologies, such as tissue engineering, it may even be possible within the laboratory to direct these cells to grow into highly organized tissues, such as arteries, for implantation into patients. Additionally, potential generation of ECs that are personalized and genetically matched with the tissues of the patient is now a possibility, and the use of these cells in allograft vessels would obviate concerns over immune system rejection of the transplant.

Stem cells represent a promising therapeutic approach that may be powerful for the treatment of cardiovascular diseases. Once we understand the detailed mechanisms of common progenitor differentiation into either ECs or SMCs, we may be able to design a new drug to direct stem cell differentiation into the cell type needed, resulting in the prevention of transplant arteriosclerosis. However, further understanding the biology of stem cells is essential to fully benefit from their regenerative properties and to design novel ways to successfully intervene with the progress of the disease.

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Disclosures

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