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Pathophysiology of Vascular Calcification in Chronic Kidney Disease

Mesenchymal Stem Cells and the Artery Wall

Angiogenesis and Pericytes in Initiation of Ectopic Calcification

Osteopontin Promoter Regulation and Phosphate Transport Molecules in Vascular Calcification

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Role of Bone Morphogenetic Proteins in Vascular Calcification

Linda Demer, Guest Editor

Mesenchymal Stem Cells and the Artery Wall

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Abstract—The presence of ectopic tissue in the diseased artery wall is evidence for the presence of multipotential stem cells in the vasculature. Mesenchymal stem cells were first identified in the marrow stroma, and they differentiate along multiple lineages giving rise to cartilage, bone, fat, muscle, and vascular tissue in vitro and in vivo. Transplantation studies show that marrow-derived mesenchymal stem cells appear to enter the circulation and engraft other tissues, including the artery wall, at sites of injury. Recent evidence indicates that mesenchymal stem cells are also present in normal artery wall and microvessels and that they also may enter the circulation, contributing to the population of circulating progenitor cells and engrafting other tissues. Thus, the artery wall is not only a destination but also a source of progenitor cells that have regenerative potential. Although potential artifacts, such as fusion, need to be taken into consideration, these new developments in vascular biology open important therapeutic avenues. A greater understanding of how mesenchymal stem cells from the bone marrow or artery wall bring about vascular regeneration and repair may lead to novel cell-based treatments for cardiovascular disease. (*Circ Res.* 2004;95:671-676.)

Key Words: vascular ■ mesenchymal stem cell ■ calcification ■ bone ■ atherosclerosis

Multilineage tissue is well known to develop in the human artery wall, especially in association with atherosclerotic lesions. First described by pathologists centuries ago, the phenomenon has been termed metaplasia, and it appears in the form of ectopic cartilage, bone, fat, and marrow.¹⁻⁶ Its significance as evidence for mesenchymal stem cells (MSCs) in the artery wall has not been appreciated until recently.

Mature bone tissue is found in 5% to 20% of atherosclerotic arteries,^{4,7} although earlier stages may occur more often.⁸ Hematopoietic marrow has been found in artery walls of 9 of 200 cases.⁴ Cartilage tissue is also found within human atherosclerotic plaque,⁹ but its frequency has not been sys-

tematically assessed. Ectopic tissue occurs in veins as well as arteries¹⁰ and in animal models of atherosclerosis.¹¹ In mice, vascular ectopic tissue is most often in the form of cartilage.^{9,12-15}

Osteogenic and chondrogenic differentiation in the artery wall are recognized clinically as vascular calcification and are detectable by x-ray, computed tomographic scanning, or ultrasound. Such mineralization is associated with increased cardiovascular morbidity and mortality.¹⁶⁻¹⁹ On the basis of physiological experiments, vascular calcification is known to increase aortic stiffness, resulting in systolic hypertension, coronary insufficiency, left ventricular hypertrophy, ischemia, and congestive heart failure.²⁰⁻²² Although plaque sur-

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rounded by a calcium deposit is under reduced mechanical stress because of load sharing, failure stress is increased at the interface of the calcium deposit with soft tissue, increasing the likelihood of tissue rupture at those edges. Approximately 85% of plaques causing coronary thrombosis are calcified.²³

Mesenchymal Stem Cells

In pioneering work, Prockop et al showed that transplanted marrow cells engraft connective tissues such as spleen and liver.^{24,25} Liechty et al showed that human marrow cells engraft multiple tissues when injected in utero in sheep.²⁶ The concept of a continuous replacement of connective tissue with marrow cells parallels the known continuous replacement of blood by bone marrow hematopoietic cells.

For hematopoietic stem cells, lineage acquisition is regulated by well-defined colony-stimulating factors. Little is known about the corresponding regulatory mechanisms for MSCs. Hematopoietic stem cells were first found in the marrow. They are now harvested from peripheral blood for clinical regeneration of marrow. MSCs were also first identified in the marrow, and they are now harvested from a variety of tissue sources.^{27–32} It has been suggested that MSCs serve as universal repair cells in adult tissues as they undergo regeneration or remodeling.^{33,34}

MSCs in Bone Marrow

Marrow stromal cells, a subpopulation of nonhematopoietic cells in the bone marrow, are the prototypical MSCs.³⁵ They represent a small percentage of marrow cells, and they can be partially distinguished from hematopoietic cells by their ability to adhere to tissue culture dishes. In culture, they may be guided to differentiate into bone, fat, cartilage, or muscle cells using specific media.^{34,36–38} When cultured in ordinary media containing 10% to 20% fetal serum, they form multiple clusters of different lineages within a single dish.³⁹ They are guided into specific, single-lineage differentiation by culture in serum-free “induction media” containing growth factors and other treatments such as insulin, dexamethasone, and indomethacin.³⁶ Recent evidence indicates that even marrow MSCs that have already fully differentiated into 1 lineage are capable of transdifferentiation into another lineage in response to induction media.⁴⁰ Marrow MSCs also maintain multipotential capacity *in vivo*, tending to differentiate into the cell type of the tissue they engraft.²⁶ This tendency to adopt local identity may be directed by local cytokines and matrix factors from host cells. Thus, adequate contact with host cells may be required to ensure that engrafted cells assume host tissue identity rather than differentiating along other mesenchymal lineages.

Importantly, the evidence that marrow MSCs transdifferentiate to adopt local tissue identity may be confounded by the phenomenon of cell fusion. In this process, a labeled donor cell fuses with a differentiated host cell, resulting in a cell positive for label and differentiation marker, falsely indicating transdifferentiation. To date, artifact attributable to cell fusion has been an issue in studies of nonvascular tissues, but in principal, it may occur in any cell type.^{41,42}

Marrow-derived MSCs also have vascular differentiation potential. In culture, they differentiate into smooth muscle

cells (SMCs).^{27,43} *In vivo*, bone marrow–derived cells that were seeded on a synthetic vascular graft produced smooth muscle and endothelial layers.⁴⁴ Although it is attractive to consider that growth of MSCs in injured vascular tissue may simply regenerate normal vascular tissue, it is also possible for these cells to produce ectopic tissues, such as those seen in advanced atherosclerotic calcification. In addition, although marrow cell–induced changes may result directly from assimilation of marrow cells, the benefit may also be an indirect, paracrine effect of cytokines released by the marrow-derived cells. The factors governing these possibilities are not clear, but it appears that metabolic diseases, such as atherosclerosis or diabetes, favor the formation of ectopic tissue.

MSCs in Arteries

The tunica media has been widely perceived as a homogeneous, terminally differentiated layer of SMCs. However, many investigators have shown that cultured SMCs are heterogeneous^{45,46} and that they undergo dedifferentiation in culture.^{47–49} Interestingly, vascular SMCs were described as “multifunctional mesenchymal cells” 36 years ago,⁵⁰ foreshadowing the finding of multipotential mesenchymal cells as subpopulations of SMCs within the artery wall.

Osteoblastic Differentiation of Vascular Cells

The first evidence that SMCs undergo osteoblastic differentiation was the production of calcified nodules by cultured microvascular pericytes.⁵¹ Similar calcified nodules were found in a subpopulation of aortic SMCs with pericyte-like features (calcifying vascular cells [CVCs]). These cells and SMCs express osteoblastic-specific genes^{52–58} in a time course characteristic of bone cell differentiation.⁵⁹ Osteoblastic differentiation also has been shown in cultures of cardiac valvular cells,^{60,61} which may account for the formation of bone in stenotic aortic valves.

Multilineage Differentiation of Vascular Cells

Pericytes and SMCs were known to have chondrogenic features,^{62,63} and their multilineage potential has now been shown *in vitro*^{54,64} and *in vivo*.⁶⁵ The lineage repertoires for the 2 cell types appear to differ slightly. Both express markers specific for chondroblasts, osteoblasts, and smooth muscle. In addition, pericytes also undergo adipogenic differentiation, whereas CVCs can undergo marrow stromal differentiation. This difference in plasticity may be attributable to technical differences or have biological significance. One possibility is that the pericytes are harvested from retinal vessels, which derive from neural crest, whereas CVCs are harvested from descending aorta, which is believed to derive from non-neural crest mesenchyme. Pericytes and CVCs are distinguished from other vascular SMCs by their expression of a surface ganglioside recognized by a monoclonal antibody (3G5).^{52,64,66} Pericyte-like cells from the human umbilical vein also form fibroblastoid cell colonies and express osteogenic, chondrogenic, and adipogenic markers.⁶⁷ It has been suggested that although pericytes have been defined by their location in the microvessels, pericyte-like cells are also present in large arteries, and they form an anatomically

continuous network in the subendothelial space.⁶⁸ Importantly, the other tissues that have been shown to contain MSCs also contain substantial vascular or microvascular tissue. This suggests the concept that pericyte-like vascular cells account for the multilineage potential in fat, skeletal muscle, and other tissues.

Regulation of Vascular Stem Cell Lineage

Factors regulating differentiation of these cells remain under investigation. Bone morphogenetic protein-2 is a candidate on the basis of its ability to regulate mesenchymal lineage in neural crest cells. This potent embryonic differentiation factor from the transforming growth factor- β superfamily is best known for promoting osteogenic differentiation, but it modulates the entire spectrum of mesenchymal lineages. Multipotent, mouse embryonic mesenchymal (C3H10T1/2) cells undergo chondrogenic differentiation at high levels of BMP-2, osteogenic differentiation at lower levels, smooth muscle or adipogenic differentiation at even lower levels, and little or no differentiation in the absence of BMP-2.^{69,70} Adult vascular MSCs organize themselves into intricate patterns in culture. These patterns are generated by a reaction-diffusion process, which is driven by the interaction of BMP-2 activity with its more rapidly diffusing inhibitor matrix Gla protein.⁷¹ The Wnt family of transcription factors, which interacts with BMP-2 in determining mesenchymal lineage in embryonic neural crest cells,⁷² may also regulate differentiation of adult vascular stem cells. These findings suggest that BMP-2 and associated factors regulate the formation of complex mesenchymal structures *in vivo*, and their control will be important in future tissue engineering.

It is not clear why MSCs undergo multilineage differentiation *in vitro*, whereas they normally remain undifferentiated or produce daughter cells that differentiate only along the same lineage as the surrounding tissue *in vivo*. In the native environment, neighboring cells or matrix may send instructive signals that are absent in culture. Extracellular matrix regulates differentiation,⁷³ and it may maintain progenitor cells in a quiescent state until the tissue architecture is disrupted. Consistent with an environmental effect, saphenous veins rarely develop calcification, but 11% of vein grafts mineralize after they are exposed to arterial circulation as coronary bypass grafts.⁷⁴ Even more rapid mineralization, in the form of cartilage and bone, occurs in mice undergoing carotid interposition surgery.⁷⁵

Engraftment

Engraftment by Marrow MSCs

In human gender-mismatched bone marrow transplant recipients, donor marrow cells engraft all layers of atherosclerotic plaques⁷⁶ but not disease-free segments, suggesting that engraftment may require inflammation or injury. Similarly, in mice with atherosclerosis, labeled donor marrow cells engraft plaque. Interestingly, recipients have less atherosclerosis when donor marrow-derived progenitor cells are from young versus older mice.⁷⁷

In transplanted hearts, a neointima forms throughout the coronaries of the transplanted heart, leading to diffuse transplant coronary arteriopathy and often ischemia. The neointi-

ma was originally believed to derive locally from the donor tunica media. However, studies in mouse cardiovascular transplant models have shown that much of the neointima derived from the host.^{78,79} When labeled veins are transplanted into the carotids of unlabeled recipient mice, the label disappears over time, suggesting engraftment by circulating host cells; conversely, unlabeled donor veins transplanted into labeled recipients gain label, again suggesting engraftment by host cells.⁸⁰ Although these results could be explained by local migration of vascular cells, the changes in labeling occurred uniformly throughout the graft rather than predominantly at the edges, supporting a circulatory origin for the engrafting cells. Because circulating cells include hematopoietic as well as mesenchymal cells, some instances of host cell "engraftment" may be attributable to ordinary diapedesis by host leukocytes, especially in areas of inflammation or injury.⁸¹ To exclude this possibility, label and phenotypic markers should be assessed in studies of mesenchymal cell engraftment.

In some experimental conditions, even injured vascular tissue is not engrafted by marrow cells.⁸² Location and type of injury may determine whether marrow cells engraft.⁸³ External injury by cuff or ligation in mice stimulates little neointimal and rare SMC layer engraftment, whereas internal injury by femoral wire denudation induces substantial neointimal (40%) and SMC (25%) engraftment.⁸⁴ Thus, technical differences may affect results of engraftment studies and need to be taken into consideration in future clinical applications.

Engraftment by Vascular Cells

Some of the first evidence for engraftment by vascular cells came from Campbell et al, who implanted a prosthetic scaffold in the peritoneum and found endothelial and SMC attachment and growth.⁸⁵ Similar engraftment was found in vascular prosthetic scaffolds.^{84,86} Recent *in vivo* studies indicate that vascular cells can emigrate from the artery wall and migrate to remote tissues, presumably through the circulation. Montfort et al transplanted aortic segments from labeled donors into the kidney capsule of recipients and found engraftment of donor vascular cells in the recipient spleen with evidence of differentiation into marrow stromal cells.⁸⁷ This important observation suggests that vascular cells may migrate from their tissue location and enter the circulation, opening possibilities for new pathophysiological mechanisms.

Although the marrow and the circulation are considered distinct compartments or sources of cells, they may overlap substantially, given that marrow-derived progenitor cells may travel in the circulation before arriving at their engraftment destination. Thus, peripheral blood mononuclear cells (PBMCs) and other circulating progenitor cells may originate from other mesenchymal tissues besides the marrow.

Endothelial Progenitor Cells

Endothelial Progenitor Cells in Circulation

PBMCs differentiate not only into lymphocytes and monocytes but also into endothelial and SMCs. When cultured on type I collagen in endothelial growth medium (EGM) with platelet-derived growth factor-BB, PBMCs express SMC

markers. Those cultured in EGM alone also develop an endothelial cell (EC) phenotype.⁸⁸ Endothelial progenitor cells (EPCs) can be isolated directly from PBMC by flow cytometric sorting using surface markers such as CD34, a marker associated previously with hematopoietic stem cells. This intriguing sharing of markers may reflect the embryonic origin of ECs from hemangioblasts, cells with hematopoietic and endothelial potential. When circulating EPCs are injected into rabbits at the time of arterial injury, they engraft the injured vascular sites.⁸⁹ EPCs promote neoangiogenesis in myocardial ischemia⁹⁰ and re-endothelialize large vessel injuries.⁹¹ Skeletal muscle tissue contains EPCs that differentiate into ECs at the site of muscle injury.⁹² Thus, EPCs may account for spontaneous regeneration of endothelium in injured or transplanted tissue.

Factors Affecting EPC Numbers

The numbers of EPCs present in the circulation are affected by lipids, estrogen status, erythropoietin, exercise, coronary risk status, and progressive cancer. Hyperlipidemia reduces the EPC numbers and engraftment potential in mice.⁸⁰ Mice treated with a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor had increased numbers of circulating cells positive for markers Sca-1 and vascular endothelial growth factor-2R.⁹³ Women with higher plasma estrogen levels have more circulating EPCs, whereas ovariectomized mice have fewer EPCs and more neointima after carotid balloon injury.⁹⁴ In patients with end-stage renal disease, treatment with erythropoietin was associated with increased numbers of CD34⁺ blood cells and increased endothelial differentiation of those cells in vitro.⁹⁵ EPC numbers, measured as colony-forming units, also correlate inversely with Framingham risk score in humans,⁹⁶ and they are increased by exercise in mice and humans.⁹⁷ Importantly, EPC numbers are also increased in the circulation in cancer patients,⁹⁸ raising the possibility that availability of EPCs may be permissive for tumor neoangiogenesis and growth.

Teleology

What would be the teleological basis for inflammation-induced ectopic differentiation of MSCs? It may be adaptive, as part of a defense against chronic infection. It is not unusual to find, deep in the middle of a soft tissue organ, a layer of bone surrounding an abscess, tuberculoma, or parasite. When leukocytes attack a pathogen, they release free radicals that oxidatively modify lipids in the pathogen membrane and surrounding tissue to a more inflammatory form.^{99,100} When the pathogen is not eliminated, these persistent inflammatory lipids may serve as a signal to local MSCs that the infection remains and triggers a more definitive immune response, such as encasement by ectopic bone to isolate the infectious process. With modern high-fat diets, hyperlipidemia results in deposition of lipoproteins in perivascular spaces of soft tissues, and these undergo partial nonenzymatic oxidation. Vascular MSCs may misinterpret these mildly oxidized lipids as evidence of persistent infectious organisms and respond by attempting to sequester them in a wall of bone or cartilage.

Summary

New evidence suggests that the artery wall is a recipient and source of MSCs. The long-recognized formation of ectopic mesenchymal tissue in the artery wall was a clue that MSCs are present in the adult artery wall. These stem cells, which were first identified in the marrow stroma, can differentiate along multiple lineages and give rise to cartilage, bone, fat, muscle, and vascular tissue in vitro and in vivo. Similar cells with multilineage and self-renewal capacity have also been harvested and cultured from muscle, blood, fat, and now adult vascular tissue. PBMCs also may serve as progenitors for endothelium and possibly smooth muscle. Marrow cells appear to enter the circulation and engraft vascular and other connective tissues, especially at sites of injury and in tissue transplant grafts. Conversely, provocative evidence suggests that cells from vascular tissue may enter the circulation and engraft remote organs. Yet, the possibility remains that MSCs from the bone marrow or the artery wall may permit human tissue regeneration and repair, an exciting future prospect for cardiovascular disease.

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