FORUM REVIEW ARTICLE

Reactive Oxygen Species Adversely Impacts Bone Marrow Microenvironment in Diabetes

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Abstract

Significance: Patients with diabetes mellitus suffer an excess of cardiovascular complications and recover worse from them as compared with their nondiabetic peers. It is well known that microangiopathy is the cause of renal damage, blindness, and heart attacks in patients with diabetes. This review highlights molecular deficits in stem cells and a supporting microenvironment, which can be traced back to oxidative stress and ultimately reduce stem cells therapeutic potential in diabetic patients. **Recent Advances:** New research has shown that increased oxidative stress contributes to inducing microangiopathy in bone marrow (BM), the tissue contained inside the bones and the main source of stem cells. These precious cells not only replace old blood cells but also exert an important reparative function after acute injuries and heart attacks. **Critical Issues:** The starvation of BM as a consequence of microangiopathy can lead to a less efficient healing in diabetic patients with ischemic complications. Furthermore, stem cells from a patient's BM are the most used in regenerative medicine trials to mend hearts damaged by heart attacks. **Future Directions:** A deeper understanding of redox signaling in BM stem cells will lead to new modalities for preserving local and systemic homeostasis and to more effective treatments of diabetic cardiovascular complications. *Antioxid. Redox Signal.* 21, 1620–1633.

Introduction

A GROWING BODY of research indicates new roles for reactive oxygen species (ROS) in health and disease. Among pathologies related to an excess of ROS, diabetes mellitus (DM) occupies a prominent position. In fact, the high associated risk for cardiovascular morbidity and mortality makes DM one of the major threats to human health in the 21 century. From 2005 to 2008, 25.8 million patients (8.3% of the population) were diagnosed with DM in the United States. An additional 79 million had impaired fasting glycemia indicative of prediabetes (12). If current trends are confirmed, the prevalence of DM among adults will reach the figure of 33% by 2050. Moreover, DM and its complications impose a public burden of economic costs (23). In 2007, the total cost of DM in the United States was estimated to be \$174 billion, \$116 billion in direct medical costs and \$58 billion in indirect costs due to disability, work loss, and premature death (12).

Cardiovascular disease (CVD), including coronary artery disease, stroke, peripheral arterial disease, and cardiomyop-

athy, are acknowledged for being the cause of death in $\approx 65\%$ of patients with DM. To make the problem worse, when patients with DM develop cardiovascular complications, they bear a poorer course compared with CVD patients without DM. One possible explanation is that healing mechanisms are dampened by the metabolic disorder. For instance, a number of studies highlight the dysfunction of resident vascular cells and circulating angiogenic cells (30, 68, 84, 92). This translates into impaired reparative angiogenesis, the process of new vessel formation by local endothelial cells (ECs) and mural cells, and vasculogenesis, which consists of recruitment and incorporation of angiogenic cells in the nascent neovasculature.

Investigation on the role of circulating angiogenic cells in CVD is complicated by the large heterogeneity of cells with direct and indirect pro-angiogenic capacities (117). Indeed, this pool includes CD34⁺ progenitor cells, Tie2 expressing monocytes, and mesenchymal stem cells (MSCs) from bone marrow (BM) and non-BM sources (26). There is, however, a consensus on the fact that circulating angiogenic cells are particularly reduced in diabetic patients who manifest vascular

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complications of the highest degree of severity (27, 28). These observations suggest a pathogenic link between the deficit in vasculogenesis-driven repair and poor prognosis of diabetic patients with CVD complications.

The reasons for the shortage of circulating angiogenic cells in patients with DM remain unclear. Different possibilities have been considered, including a general reduction in hematopoietic stem cells (HSCs) or a defect in HSCs becoming monocytes or other progenitors within the BM, a reduction in circulating monocytes, or a specific incapacity of monocytes to become circulating angiogenic cells. In this regard, recent studies suggest that molecular modifications caused by chronic hyperglycemia might endanger stem cells and their progeny, that is, lineage committed progenitors, in their primitive niches (91). Furthermore, the lack in progenitor cell mobilization and preferential differentiation toward a pro-inflammatory phenotype have been reported in patients with DM (25, 41, 70, 84, 113). Importantly, the possibility that disruption of the normal redox balance participates in the damage of BM stem cells and their supportive microenvironment is gaining much attention.

In this review, we illustrate current knowledge of the mechanisms by which DM impinges on stem cell functions, including survival, self-renewal, differentiation, proliferation, migration, and mobilization. We also focus on DM as a disease model in which excessive ROS formation can jeopardize stem and stromal cells of the marrow niche. Advancing our understanding on how disruption of the redox balance induces excessive stimulation of stem cell differentiation, activation of apoptosis and, eventually, exhaustion of BM regenerative capacity is fundamental for establishing preventive measures in patients at risk of cardiovascular complications. It is also hoped that research will provide a new scope for preservation of BM integrity during normal and pathologic aging. Furthermore, managing ROS levels could be a rational means for better utilization of stem and progenitor cells in supply-side cell therapies of regenerative medicine for patients with manifested CVD.

Organization of BM Microenvironment

HSCs were the first adult stem cells employed for therapeutic purposes. The use of BM stem cells has recently expanded from treatment of hematologic disorders to initial clinical trials of cardiovascular regenerative medicine. Along with an increasing number of clinical applications, knowledge is growing on the presence of different functional compartments for stem cell residence. In the BM, stem and progenitor cells are distributed in distinct microenvironments called “niches” (93) constituted by a frame of stromal cells, macrophages, regulatory T cells, and the extracellular matrix (33, 72, 115). Two distinct niches have been identified: the *osteoblastic niche* (also named *endosteal niche*) lining on the endosteal bone surface and the *vascular niche* located near the sinusoids, which constitute the vast majority and most peculiar component of BM vasculature (Fig. 1). The existence of two niches creates a polarized gradient for stem cell self-renewal, maturation, and relocation into the systemic circulation. Several vascular sinusoids are recognized in close proximity to the endosteum, which indicates an intricate relationship between the two niches (86).

Heterogeneity of BM Perfusion, Oxygenation, and ROS Content Creates Specialized Areas for Stem Cell Self-Renewal and Differentiation

It is now becoming increasingly evident that the HSC compartment consists of several subpopulations with distinct expansion and differentiation behaviors. This heterogeneity is attributed to the existence of stem cell-intrinsic programs under the influence of different milieus (74). One major milieu element is represented by the peculiar gradient of perfusion and oxygenation across the marrow (79, 81). The presence of high and low oxygenic areas creates the conditions for a differential production of ROS in different niches. In recent years, the interest on ROS as a key determinant of the marrow microenvironment heterogeneity has significantly increased, especially in the light of its recognition as a second messenger in growth factor-mediated angiogenic signaling and hypoxia-induced mobilization of angiogenic cells (88, 89) as well as a stimulus skewing the balance away from stem cell self-renewal toward differentiation (50, 103). In line with this, ROS levels are higher in terminally differentiated ECs than in endothelial progenitors (17). Furthermore, the low oxygenic, low ROS zone abundantly contains undifferentiated cells with high self-renewal potential (Fig. 1) (50, 75, 83, 96, 97). Conversely, the relatively high ROS concentration found in the vascular niche is instrumental to stem cell maturation (58).

Intracellular levels of ROS are important not only to instruct BM cell behavior but also for determining cell relocation (56, 57) and egression into the bloodstream (38, 51, 85, 99). Importantly, ROS play a key role in BM mononuclear cell (MNC) and angiogenic cell mobilization on induction of peripheral ischemia in experimental models (109). Thus, ROS acts as a second messenger that facilitates stress-induced mobilization of stem and progenitor cells as a part of host defense and repair mechanisms.

Sources of ROS and ROS-Mediated Mechanisms

Although the role of ROS in regulation of stem cell biology is well acknowledged, it is not yet completely clear “who-does-what” among different types of ROS. The short half life and easy diffusion of ROS along with limitations of many dyes currently used for measuring ROS represent an obstacle to mechanistic understanding (53). On the other hand, it is possible to reconstruct the physiological scenario by studying the different sources of ROS and downstream pathways.

ROS generators are mainly represented by the mitochondria and NADPH oxidase machinery. In mitochondria, ROS are a by-product of the respiratory chain enzymes complex I and complex III. The tuberous sclerosis complex (TSC)—mammalian target of rapamycin (mTOR) pathway maintains the quiescence of HSCs by repressing mitochondrial biogenesis and the local production of ROS. Conversely, mitochondrial generation of ROS drives HSCs from quiescence into rapid cycling (14). Further supporting the link between mitochondrial ROS and stem cell biology, Liu *et al.* showed that BM cells from polycomb repressor *Bmi-1* knock-out mice have mitochondrial dysfunction, reduced ATP, increased ROS levels, and subsequent DNA damage (66) Of note, mitochondrial DNA is particularly susceptible to oxidative stress because of its vicinity to the electron transport chain and also due to the fact that it lacks protective histones.

FIG. 1. BM microenvironment organization. The BM is spatially organized into different niches. The osteoblastic niche (*left*) resides in the inner part of BM, lining on the endosteum. It includes the most primitive stem cells and a frame of stromal cells, principally osteoblasts. The vascular niche (*right*) is constituted by committed progenitor elements that are embedded in a frame of several kinds of stromal cells (ECs, macrophages, pericytes, *etc.*). In both niches, stem and progenitor cells establish cell–cell contacts that are instrumental to reciprocal control of cell function. Ang-1, angiopoietin 1; BM, bone marrow; cKit, stem cell factor receptor; EC, endothelial cell; MMP-9, metalloproteinase 9; N-Cad, N-cadherin; SCF, also known as kit-ligand, stem cell factor; SDF-1, stromal cell-derived factor-1; SDF-1 receptor, CXCR4, C-X-C chemokine receptor type 4; Tie2, angiopoietin receptor 2; VCAM-1, integrin receptor vascular cell adhesion molecule-1; very late antigen-4, V α 4, integrin α 4 β 1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Accumulation of damage in the mitochondrial genome contributes to cellular aging. Accordingly, mutations of mitochondrial DNA accelerate the stem cell functional decline by influencing mobilization of energy and the balance between production and disposal of mitochondrial-derived ROS (4, 36).

NADPH oxidases are a family of enzymes that catalyze the transfer of electrons from NADPH to molecular oxygen *via* their “NOX” catalytic subunit, thereby generating superoxide and hydrogen peroxide and controlling numerous biological and pathological processes (8, 61). Among NOX isoforms, NOX1 is expressed in vascular smooth muscle cells and other vascular cells. NOX2, previously known as gp91phox, is present in endothelial and phagocytic cells (39, 105). NOX4 is constitutively expressed in ECs, vascular smooth muscle cells, cardiac cells, and phagocytic cells (3, 44). HSCs express NOXs 1, 2, and 4, which generate low levels of ROS to regulate cell growth and differentiation (82). NOX2-derived ROS is involved in ischemia-induced mobilization of and neovascularization afforded by progenitor cells and circulating angiogenic cells (88, 109). Thus, NOXs play a pivotal role in maintaining ROS levels within a limited range in order to regulate physiological functions of BM stem and progenitor cells and circulating angiogenic cells.

Molecular Pathways Involved in ROS Signaling

There are many downstream actors involved in regulation of the BM microenvironment and stem cell biology by ROS (Fig. 2). Of course, cells of the vascular niche and HSCs have

different ROS signaling mechanisms and targets. In vascular cells, ROS are responsible for modulation of angiogenesis and vascular permeability as well as for cell-extrinsic regulation of stem cell maturation (*vide infra*); whereas, in HSCs, ROS signaling participates in cell-intrinsic regulation of hematopoiesis. Recent evidence from studies in genetically modified animals indicates that ROS is a key modulator of the PI3K–Akt signaling pathway in HSCs. In 2010, Juntilla *et al.* investigated physiologic hematopoiesis in *Akt1*^{−/−}/*Akt2*^{−/−} mice. Interestingly, serial transplantation experiments suggested that Akt is important for maintaining long-term HSCs throughout generation of ROS (52). Moreover, Kharas *et al.* showed that rapid induction of Akt signaling causes expansion of the HSCs compartment and increased HSC cycling, whereas a sustained Akt signaling in myr-AKT mice causes depletion of HSCs and increased apoptosis, eventually resulting in myeloproliferative disorders (55). Phosphatase and tensin homolog (PTEN) is a negative regulator of the PI3K–Akt pathway; thus, it does not come as a surprise that *PTEN*^{−/−} mice show a phenotype similar to that of animals with activated Akt (122). The effects of PTEN are, in part, dependent on ROS production in HSCs. In addition, downstream inhibitory targets of Akt, such as the forkhead box transcription factors (FOXOs), are critical mediators of the cellular responses to oxidative stress in HSCs (95). In particular, mice lacking *FOXO3a* show altered HSC proliferation and differentiation patterns, with increased susceptibility to damage induced by stress and myeloid bias with aging, a defect associated to increased ROS level and p38 mitogen-activated protein kinase (MAPK) phosphorylation

FIG. 2. Oxidative stress network in BM microenvironment. In BM, the sources of ROS are principally represented by the mitochondria and NADPH oxidase catalytic subunit NOX. Superoxide produced is then converted by superoxide dismutase into hydrogen peroxide and scavenged by catalase or glutathione peroxidase. ROS can enhance or decrease stem cell functions by activating or inhibiting different signaling pathways. For example, ROS induce differentiation and migration through the PTEN/PI3K/Akt pathway, sustain quiescence *via* FOXOs and ATM, and induce senescence through p38 MAPK pathway. ATM, ataxia telangiectasia mutated protein; FOXOs, forkhead box transcription factors; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(73, 104). Furthermore, activation of p38 MAPK in response to increasing levels of ROS limits the lifespan of HSCs *in vivo* (49). Accordingly, *FOXO3a* deficiency is counterbalanced by antioxidant treatment with *N*-acetylcysteine (104).

The nuclear factor erythroid-2-related factor 2 (Nrf2), a master regulator of the antioxidant response, is required for maintenance of HSC homeostasis with a mechanism partially independent of ROS levels (45, 107). Along with FOXO3a and Nrf2, the *ataxia telangiectasia mutated* (*ATM*) gene exerts a protective role in HSCs against oxidative stress, as it maintains genomic stability by activating cell-cycle checkpoint, as demonstrated in *ATM*^{-/-} mice (2, 48, 64). Similar to *FOXO3a*^{-/-} mice, antioxidant treatment rescues HSC alteration in *ATM*^{-/-} mice (48). Lastly, another factor regulating HSCs quiescence is the tumor suppressor gene p53. HSCs from mouse *double minute 2 homolog* (*Mdm2*, an ubiquitin ligase that targets p53 for degradation) knockout mice are characterized by elevated ROS, cell cycle arrest, senescence, and cell death in the hematopoietic compartment. This phenotype is partially rescued by antioxidants (1).

Although studies from genetically modified mice are certainly informative, it should be noted that the complete ablation of a specific pathway might not reflect the real pathophysiologic situation. Furthermore, the pool of HSCs is heterogeneous and, therefore, alteration of ROS levels in distinct populations could significantly change the scenario predicted by studies in which total BM cells carry a genetic mutation responsible for redox unbalance.

Implication of ROS in Physiological Aging of BM Stem Cells

Although HSCs can persist *in vivo* longer than a whole organism's life-span, the hematopoietic compartment is not spared by aging, which results in reduced stem cell regenerative potential, diminished adaptive immune competence, myeloid bias, and myeloproliferative disease predisposition. Among different factors responsible for stem cell senescence, genomic DNA damage accrual and epigenetic modification of chromatin components are thought to limit the regenerative response of HSCs from old mice (9, 43). Generalized expansion of the high ROS zone to the whole marrow may also contribute to the unbalance between self-renewal and differentiation in aging individuals.

Excessive ROS Production in Diabetes

Vascular ECs are particularly exposed to damage, because they are unable to downregulate the uptake of glucose when extracellular glucose concentrations are elevated. The high levels of intracellular glucose cause mitochondrial superoxide overproduction (106, 121). In turn, superoxide induces the activation of five major pathways involved in the pathogenesis of complications: polyol pathway flux, increased formation of advanced glycation end-products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway (21). Increased ROS production in the mitochondria causes DNA strand breaks

and mutations, activating poly(ADP-ribose) polymerase (PARP), which, in turn, is responsible for further stimulation of toxic metabolic pathways (21).

In addition to the mitochondria, NADPH oxidase has been credited as one of the main sources of ROS production in DM. Hyperglycemia triggers PKC- α -induced NADPH oxidase activation *via* intracellular AGE formation (100). A sustained NADPH oxidase activation may lead to exhaustion of intracellular NADPH reservoir, which is fundamental for the activity of endothelial nitric oxide synthase (eNOS) and several antioxidant systems. Thus, NADPH oxidase could work as a double-edged sword, with transient activation providing a feedback against excessive ROS generation through the activation of antioxidant redox-sensitive Nrf2-Keap1 signaling pathway. In DM, this balanced mechanism seems to be disrupted: ROS overproduction leads to eNOS uncoupling, mitochondrial dysfunction, and impaired antioxidant defenses, resulting in depletion of intracellular NADPH (35).

Microangiopathy in BM of Diabetic Mice Jeopardizes Stem Cell Homeostasis

The vascular system pervades all organs of the body. Hence, the systemic nature of diabetic damage is strictly related to microangiopathy, which, along with diffuse ath-

erosclerotic disease, represents the main feature of diabetic vasculopathy. While the consequences of microangiopathy for the retina, heart, brain, kidneys, and lower extremities are very well known, no specific research was performed until recently about the BM microvasculature and the possible impact of diabetic microangiopathy on SC homeostasis.

There are good reasons for considering the vascular niche as a primary site of increased oxidative stress in diabetic BM. This is a high oxygenic region compared with low perfused endosteum. In addition, DM could primarily increase oxidative stress in ECs because of lack of control in glucose influx in these cells. Accordingly, we found that ROS levels are already increased in BM ECs at 14 weeks after induction of type 1 DM (69), whereas oxidative stress was extended to HSCs after ~20 weeks of DM in the same animal model (79). However, to the best of our knowledge, no study has addressed the issue of whether DM increases ROS levels in HSCs directly or through mediation of cells of the vascular niche.

We were the first to demonstrate that diabetic microangiopathy in streptozotocin (STZ)-induced type 1 diabetic (T1D) mice might be the cause of BM dysfunction through the creation of an hostile environment where stem cells, namely Lin⁻/Sca-1⁺/c-Kit⁺ (LSK) cells, undergo oxidative stress-induced damage, accelerated senescence, and death by apoptosis (Fig. 3) (79). The LSK cell population showed a

FIG. 3. Proposed model of microenvironmental alteration in diabetic BM. Representation of endosteal and vascular niches in healthy and diabetic conditions. Different colors identify HSCs (blue), HPCs (green), MSCs (beige), and ECs (brown). Erythrocytes inside the marrow vessels are represented in red. Excessive production of ROS in the marrow microenvironment tends to cause a disruption of stem and progenitor cell homeostasis. The ROS gradient is lost, thus triggering stem cell proliferation and differentiation in areas of the marrow that are devoted to maintaining stem cell quiescence. Furthermore, failure of sinusoidal barrier function causes unselective mobilization, skewed toward inflammatory cells instead of regenerative cells. In addition, microvascular rarefaction causes hypoperfusion and deprives stem cells of necessary trophic inputs, leading to stem cells apoptosis. Altogether, these changes jeopardize the regenerative capacity of BM with consequences for peripheral complications. HPCs, hematopoietic progenitor cells; HSCs, hematopoietic stem cells; MSCs, mesenchymal stem cells. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

greater ROS content assessed by CM-H₂DCFDA. Likewise, BM MNCs isolated from trabecular bone showed an increased oxidative stress at mitochondrial level. Oxidative stress causes DNA damage, thereby reducing the lifespan of BM stem cells. In line, the levels of phosphorylated histone H2AX, a marker of double DNA strand breaks, were 2.5-fold higher in BM cells from T1D mice compared with nondiabetic age-matched controls. Since H2AX is phosphorylated by ATM, we analyzed the expression in T1D BM cells and found that this gene is 2.6-fold upregulated compared with BM cells of nondiabetic mice. Furthermore, flow cytometry analysis of Annexin V-positive cells unraveled the increased apoptosis of LSK cells from BM of T1D mice.

What is the link between microangiopathy and BM cell damage and does BM remodeling contribute to peripheral complications? We found that vascular density is drastically decreased in the BM of diabetic mice at the capillary, sinusoid, and arteriole level. Microvascular rarefaction was associated to a profound reduction of the marrow perfusion as assessed by fluorescent microspheres. We next determined the relative position of LSK cells with regard to *in vivo* Hoe dye perfusion gradient and the distribution of MECA32⁺ ECs. Hoe was injected intravenously and then, the degree of uptake of the dye by BM cells from different locations was evaluated by flow cytometry. We found that the Hoe^{low} perfusion region contained 53% of total LSK cells in BM of nondiabetic mice, but this fraction was reduced to 21% in T1D BM. Reversing the gating procedure, we analyzed the abundance of LSK cells in total cells and lympho-monocyte fraction of each Hoe perfusion area. Results showed that LSK cells were depleted in the low-perfused zone of T1D BM, but preserved in the high-perfused zone, which corresponds to the predominant localization of MECA32⁺ BM ECs (*e.g.*, the vascular niche). Altogether, these data suggest that the hypo-perfusion caused by contraction of the vascular niche is a major determinant of stem cell reduction in BM of diabetic mice. However, in a similar study conducted by Orlandi *et al.* in C57BL/6 STZ-induced diabetic mice, no microvascular rarefaction was observed, probably due to the different mouse strain analyzed and shorter duration of DM (80).

Microvascular rarefaction, reduction of the hematopoietic fraction, and adiposity were also observed by us in BM of leptin receptor transgenic mice, which develop obesity and type 2 diabetes (T2D) as they reach adulthood. The adipose tissue deposition in BM of diabetic mice merits attention. Interestingly, oxidative stress is a major factor impairing MSC function, resulting in decreased osteogenesis in favor of adipogenesis (7). MSCs differentiate into osteoblasts or adipocytes according to multiple signaling pathways, including those influenced by heme oxygenase-1 and -2 (111). Adipose tissue not only replenishes the space left by hematopoietic tissue shrinkage but may also act as a negative regulator of the BM microenvironment through secretion of pro-inflammatory cytokines (77).

Oxidative Stress Causes Endothelial Barrier Dysfunction in Diabetic BM

Besides providing perfusion and oxygenation, the vascular niche exerts trophic support to cells of the niche and acts as a gatekeeper for passage of cells and endocrine factors from and to the peripheral circulation.

Therefore, we have investigated the status of BM endothelium by *in vivo* assessment of vascular permeability, *in vitro* fluorescent-labeled dextrans, and we found an increased vascular permeability in BM of diabetic mice. Furthermore, BM ECs isolated from T1D mice showed a variety of functional alterations, including diminished production of factors that exert trophic actions on hematopoietic cells, impairment of migration and network formation capacity, and altered barrier function. The latter manifested as increased permeability to macromolecules and spontaneous trans-endothelial migration of BM MNCs, but reduced directed trans-endothelial migration of the same cells toward chemoattractants. The analysis of differentially expressed transcripts in BM ECs from T1D and nondiabetic mice showed an effect of DM on signaling pathways controlling cell death, migration, and cytoskeletal rearrangement. We also found a remarkable increase in mitochondrial ROS levels in diabetic endothelium. Increased oxidative stress was associated to upregulation of PARP and downregulation of Nfr2. It is known that oxidative stress induces DNA strain breaks, which, in turn, activate PARP; whereas Nfr2 exerts antioxidant activity to protect vascular cells from glucose-induced damage. In contrast, the expression of NADPH oxidase isoform 2, another important source of ROS, was similar in BM ECs from diabetic and control mice. Altogether, these data suggest that oxidative stress in BM endothelium is attributable to increased ROS production in mitochondria and reduced antioxidant defense.

Redox-dependent activation of small guanosinetriphosphatases (GTPases), including RhoA and its associated protein kinases ROCK1/ROCK2, is implicated in DM-induced endothelial dysfunction (90, 110). PKC is reportedly implicated in RhoA activation by ROS (13, 22, 112). Using a RhoA–GTP-bound pulldown assay, we documented that DM increases Rho activity in BM ECs. Importantly, ROS scavengers inhibit RhoA activation in this cellular setting. Moreover, both RhoA knocking down with a dominant negative form and ROCK inhibition rescued endothelial dysfunction, restoring Akt-dependent production of angiocrine factors, vascular permeability, and trans-endothelial migration of BM MNCs.

Small GTPases are not the only determinants of altered vascular permeability in BM of diabetic mice. We found that diabetic BM ECs have a higher phosphorylation levels of VE-cadherin at tyrosine 731, which is the binding site for β -catenin, and proline rich kinase 2 (Pyk2) at tyrosine 402 (*i.e.*, its auto-phosphorylation site). Phosphorylation of VE-cadherin and Pyk2 favors the disassembly of adherens junctions and BM MNC extravasation (69). Along with Pyk2, DM increases the activity of other kinases, including p38, JNK, MEK1, and ERK1/2, which regulate EC viability, proliferation, and barrier function (120).

In summary, BM-specific endothelial dysfunction may play a relevant role in diabetic complications. In fact, microvascular rarefaction damages BM stem cells through reduction of perfusion and suspension of paracrine trophic signaling (58). Increased production of ROS in ECs may transfer oxidative stress to other cellular components of the niche, thereby propagating cellular damage to HSCs (69, 79). On the other hand, interaction of HSCs with stromal cells facilitates the ROS flux between neighboring cells, thus reducing the negative effect of excessive ROS production

through redistribution. For instance, HSCs can prevent senescence by transferring oxidative stress to stromal cells through gap junction's component connexins-43 (47, 98).

ROS is a key determinant in causing the failure of the BM endothelium to modulate the regular passage of macromolecules and cells (11, 32). The loss of barrier function leads to plasma extravasation and increased interstitial pressure, which are particularly harmful for tissues such as the marrow contained in nonexpandable bone (Fig. 3). Altered vascular barrier function has negative consequences for regulated stem cell liberation as confirmed by studies of diabetic mobilopathy (*vide infra*).

Studies of Human BM Indicate Remodeling of the Vascular Niche

Although the pauperizing effect of DM on circulating angiogenic cells is well recognized, investigation on BM of diabetic patients is limited to aspirate samples. By a flow cytometry approach, Fadini *et al.* showed a decrease in the abundance of CD34⁺ cells in a group of 10 diabetic patients and 10 nondiabetic controls (24). A retrospective evaluation of BM biopsies from patients with suspected hematologic disorders confirms the depletion and impaired mobilization capacity of BM in DM. Furthermore, diabetic patients undergoing BM transplantation showed slow or unsuccessful engraftment compared with nondiabetic patients (31). These data suggest that DM reduces the regenerative capacity of BM stem cells but do not clarify whether the delayed engraftment was also due to damage of the niche.

We recently performed a case-control study in large cohorts of T2D diabetic subjects and nondiabetic controls without suspect of hematologic disorders, who were recruited on the occasion of orthopedic surgery. In parallel, we investigated the BM of T2D patients undergoing amputation for critical limb ischemia (CLI) (91). Employing both flow cytometry of isolated BM cells and *in situ* immunofluorescence staining, we showed the depletion of hematopoietic and pro-angiogenic progenitors, namely CD34⁺, CD34/CD133⁺, CD34/KDR⁺, and CD34/CD14/KDR/CXCR4⁺ cells, but not of mature hematopoietic cells. Moreover, *in situ* detection of DNA fragmentation by terminal deoxy nucleotidyl transferase UTP nick end labeling assay indicates activation of apoptosis in BM CD34⁺ cells of diabetic patients compared with controls. The marrow was replaced by accumulating fat, especially in patients with CLI. Notably, we also demonstrated the presence of microangiopathy in BM of diabetic patients, thus confirming our earlier findings in T1D mice. We found that both microvascular rarefaction and depletion of CD45^{dim}CD34⁺ cells in BM could be predicted by duration of DM and fasting glucose in a multiple regression analysis. Therefore, we speculate that the failing microvasculature, which feeds the marrow niche, could reduce the production of progenitor cells, including pro-angiogenic cells. Nonetheless, prospective studies are required to verify the cause-effect relationship between these correlates.

DM Impacts Stem Cell Viability by Inhibiting Hematopoietic microRNA

High glucose could damage BM stem cells directly by upregulating a pro-apoptosis pathway. In fact, investigation of underpinning mechanisms revealed that DM impacts

microRNAs (miRs), which are small RNA molecules controlling gene expression and hence biological functions. In particular, miR-155 is drastically reduced in CD34⁺ progenitor cells from diabetic patients. The miR-155 is implicated in maintenance of BM cell stemness, by holding HSCs at an early stem-progenitor stage through inhibition of differentiation-associated molecules, such as CCAAT/enhancer-binding protein- β , cAMP response element-binding protein, JUN, and FOS (37, 71).

In addition, we found that miR-155 downregulation is associated to induction and nuclear localization of FOXO3a in BM stem cells of diabetic patients and consensual upregulation of p27^{Kip1}. Likewise, *in vitro* exposure of healthy CD34⁺ cells to high glucose reproduced the transcriptional changes induced by DM, with this effect being reversed by forced expression of miR-155. How to reconcile these data with results from genetically modified mice showing that the abrogation of FOXO3a results in increased susceptibility to stress-induced damage of BM stem cells? One possible explanation is that in hematopoietic cells with incurred DNA damage, FOXO3a induces cell cycle arrest and apoptosis, *via* transcriptional regulation of the cyclin-dependent kinase inhibitor p27^{Kip1} and pro-apoptotic Bcl-2 family member Bim (59, 62, 94, 119). Thus, by inhibiting miR-155, DM might exert a negative impact on HSC survival. It should be noted that our study provides an instantaneous picture of the histological and molecular alterations incurred by the BM of patients with long-term DM, but does not allow us to draw a conclusion on the time course of these features.

These data are intriguing, as increasing evidence points to miRs as master regulators of hematopoietic cell functions (37). In this regard, high levels of miR-155 have been recently found in circulating CD34⁺ cells after stimulation of mobilization by systemic chemokine administration in healthy nonhuman primates (20). Furthermore, miR-155 confers lymphoma cells and splenocytes with increased migratory activity in response to stromal cell-derived factor-1 (SDF-1) (15). Therefore, miR-155 downregulation might contribute to the defective mobilization of stem cells in patients with DM. Additional investigation is needed to elucidate whether miR-155 is under control of ROS and whether ROS can affect stem cells and cells of the niche through modulation of other redox-sensitive miRs. A recent study showed that short-term stimulation of cardiac stem cells with hydrogen-peroxide induces miR-155, which, in turn, attenuates necrotic cell death by targeting receptor interacting protein 1 (RIP1) (67). However, it is not clear how the acute ROS elevation activates miR-155 and whether this represents a reactive defense response that can be overwhelmed by persistent oxidative stress. Another investigation showed that ROS inhibits miR-199a and miR-125b expression through increasing the promoter methylation of the miR-199a and miR-125b genes by DNA methyltransferase 1 (42). The generation of miRs is dependent on the RNase III enzyme Dicer that cleaves the pre-miR transported from the nucleus to a ~22 nt duplex miR. Interestingly, Dicer is inhibited by multiple stresses, including ROS, phorbol esters, and the Ras oncogene (114). In addition, ROS can induce direct oxidative inactivation of mRNAs and miRs. Thus, ROS could influence miR activity *via* epigenetic changes influencing their transcription, maturation, and processing (Fig. 4).

FIG. 4. Influence of ROS on microRNA generation. Recent evidence indicates that miRs control several functions of stem cells. ROS can influence the regulation of gene expression through interference of miRs. This influence is exerted at different levels of miR generation and processing. miR, microRNA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Diabetic Mobilopathy

HSCs detach from BM niches and mobilize into circulation. Recent studies demonstrate the defective mobilization of LSK cells in rodent models of DM (16, 29). Similarly, diabetic patients show impaired mobilization of stem cells after stimulation with granulocyte colony-stimulating factor. The new term “mobilopathy” was coined to illustrate this DM-induced stem cell deficit (Fig. 5) (19, 31). Stem cell

mobilization is mediated by several cytokines, growth factors, and hormones and is dependent on the intrinsic motility of stem cells and permeability of the endothelial barrier, both of which are potentially influenced by ROS levels. Stem cells isolated from murine diabetic BM show eNOS uncoupled and inactivation (101). This nitric oxide generating enzyme is crucial for proper mobilization. Hence, its inactivation might account for diabetic mobilopathy. Interestingly, one of the major causes of eNOS uncoupling is represented by sustained

FIG. 5. Diabetes-induced mobilopathy. Stem and progenitor cell mobilization from BM is a multistep process triggered by a gradient of chemokines generated from ischemic tissues. Chemokines also promote proteolytic activity in the marrow environment, thus favoring stem and progenitor cell relocation from the endosteal niche to the vascular niche and then the passage to the bloodstream. Oxidative stress generated in stem cells by activation of NOX2 acts as a mediator of those chemokines. However, sustained and excessive oxidative stress can lead to eNOS uncoupling, thereby compromising stem cell mobilization. eNOS, endothelial nitric oxide synthase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

oxidative stress generated by NADPH oxidase (63). Conversely, several growth factors trigger progenitor cell mobilization *via* NOX2 (88). In particular, the SDF-1/CXCR4 duo is the most important axis in regulating HSC mobilization according to circadian fluctuations (87). Those fluctuations are in anti-phase with SDF-1 levels in the BM and are strongly affected by light exposure or “jet lag.” Autonomic noradrenergic fibers regulate stem cell release and expression of SDF-1 (87). In fact, the BM niche is innervated by an abundant plexus of noradrenergic and peptidergic fibers, which reach the different niche elements (76). Furthermore, nociceptive signaling from ischemic organs can activate the release of pro-angiogenic cells from the BM (5). Osteoclasts have been demonstrated to be necessary for HSC mobilization. It is known that ROS is a potent inducer for differentiation of monocytes into osteoclasts (118).

Altogether, these data indicate that ROS is necessary for proper mobilization. Disruption of the redox balance might result in disordered mobilization in response to peripheral stimuli.

Therapeutic Perspectives

The quest for treatment of BM damage induced by DM is challenging. A tight control of glycemia is certainly useful as documented by us in a T1D diabetic model using insulin implants, which resulted in prevention of BM microangiopathy and remodeling (69). Furthermore, since ROS is implicated in these processes, the use of ROS scavengers seems to be also obvious (46, 65, 123). However, a recent meta-analysis of 68 randomized trials showed no evidence of benefit on mortality, but, rather surprisingly, an increased risk of death by several common anti-oxidant dietary supplements (10). Alternatively, increasing the activity of anti-oxidant enzymes, such as the use of superoxide dismutase (SOD) mimetics, is being considered (78). Of course, the treatment should be carefully designed, in order to match the correct range of ROS levels necessary for physiological stem cell functions (40, 102, 108). Conversely, short-term ROS supplementation could be useful in acute situations to boost reparative angiogenesis in ischemia. *Ex vivo* preconditioning of stem cells with ROS could also reinvestigate their regenerative potential before transplantation into ischemic tissues (18, 60).

Systemic administration of agents that are able to contain chronic oxidative stress by interfering with key steps of the metabolic disorder could be considered. Benfotiamine, a vitamin B1 derivative, blunts the consequences of oxidative stress by diverting the excess of glucose toward the pentose pathway. Interestingly enough, benfotiamine proved to prevent stem cell depletion in BM (79) and hearts of diabetic mice (54), while concurrently exerting therapeutic effects of peripheral microvascular complications (34). Similarly, statins are attractive for their pleiotropic action, which include the preservation of pro-angiogenic cells in DM (6). Statins treatment improves stem cell mobilization and endothelial commitment (6). In addition, statin could have a direct effect on BM endothelium and niche-resident stem cells through its inhibitory effect on the Rho-ROCK pathway (69).

Finally, physical exercise training has been reported to improve clinical outcome, and, thus, it has been implemented into care guidelines to cardiovascular patients. The relevance of regular physical exercise for maintenance of BM fitness is

seemingly due to an increased expression and activity of eNOS, as well as to a lower expression of NADPH oxidase in angiogenic cells (116).

Conclusions

The role of ROS in regulation of stem cell biology is firmly established. We foresee that a deeper understanding of redox signaling in those precious cells will translate soon into new modalities for preserving local and systemic homeostasis as well as into safer and more effective treatments of diabetic cardiovascular complications.

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Abbreviations Used

AGE = advanced glycation end-product
 Ang-1 = angiopoietin 1
 ATM = ataxia telangiectasia mutated
 BM = bone marrow
 CLI = critical limb ischemia
 CVD = cardiovascular disease

CXCR4 = C-X-C chemokine receptor type 4
 DM = diabetes mellitus
 EC = endothelial cell
 eNOS = endothelial nitric oxide synthase
 FOXO3A = forkhead box, subclass O3a
 GTPases = guanosinetriphosphatases
 HSC = hematopoietic stem cell
 LSK = Lin⁻/Sca-1⁺/c-Kit⁺
 MAPK = mitogen-activated protein kinase
 Mdm2 = mouse double minute 2 homolog
 miRs = microRNAs
 MMP-9 = metalloproteinase 9
 MNC = mononuclear cells
 MSC = mesenchymal stem cell
 mTOR = mammalian target of rapamycin
 N-Cad = N-cadherin
 Nrf2 = nuclear factor erythroid-2-related factor 2
 PARP = poly(ADP-ribose) polymerase
 PI3K = phosphatidylinositol 3-kinase
 PTEN = phosphatase and tensin homolog
 Pyk2 = proline rich kinase 2
 RIP1 = receptor interacting protein 1
 ROS = reactive oxygen species
 SCF = stem cell factor
 SDF-1 = stromal cell-derived factor-1
 SOD = superoxide dismutase
 STZ = streptozotocin
 T1D = type 1 diabetes
 T2D = type 2 diabetes
 Tie2 = angiopoietin receptor 2
 TSC = tuberous sclerosis complex
 VCAM-1 = integrin receptor vascular cell adhesion molecule-1
 V α -4 = very late antigen-4/integrin α 4 β 1
 VE-cadherin = vascular endothelial-cadherin