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Bone Marrow as a Target and Accomplice of Vascular Complications in Diabetes

Running title: Bone marrow and ischemia

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Abstract

Peripheral vascular complications are common in diabetic patients. While pathogenic mechanisms have received much consideration, only recently regenerative processes captured attention. There is now a consensus that the bone marrow is a source of reparative cells and that this healing mechanism is lost in people with diabetes, especially in those suffering from ischemic complications. This failure was thought to occur due to a negative impact of diabetes on the mobilization of stem/progenitor cells with angiogenic properties from the bone marrow to the circulation. Moreover, those patients showing severely reduced bone marrow cell mobilization also bared a very high risk for adverse cardiovascular events. More recently, the structural integrity of the bone marrow was recognized to be altered because of the rarefaction of local microvasculature and innervation, thus mirroring anatomical features that typically occur in peripheral tissues. Ensuing hypoxia, nutrient starvation, and creation of an acidic and oxidative environment concur in causing the depletion of stem/progenitor cells in the endosteal niche and in forcing stromal cells to activate an adipogenesis program. Moreover, stem/progenitor cells acquire a pathogenic phenotype and, once mobilized, can pass harmful signalling molecules to vascular cells of peripheral tissues thereby contributing to ischemic complications. These new pieces of evidence indicate that the bone marrow should deserve more attention in the current care of critical limb ischemia and diabetic foot. Owing to powerful reserve capacities, the bone marrow integrity could be preserved and even rescued using rehabilitation programs and pharmacological treatments with consequent benefit for local and whole-organism homeostasis.

Keywords: Bone marrow, ischemia, diabetes, microangiopathy, stem cells

Abbreviations:
bone marrow (BM)
critical limb-threatening ischemia (CLTI)
diabetes mellitus (DM)
endothelial progenitor cells (EPCs)
granulocyte-colony stimulating factor (G-CSF)
hematopoietic stem/progenitor cells (HSPCs)
mammalian target of rapamycin (TOR)
monocyte chemoattractant protein-1/chemokine (C–C motif) ligand-2 (MCP-1/CCL2)
nerve growth factor (NGF)
peripheral artery disease (PAD)
osteoclasts (OCLs)
progenitor cells (PCs)
silent information regulator (SIR)T1
substance-P (SP)
stem cells (SCs)
transient receptor potential cation channel subfamily V member 1 (TRPV1)
tumour-necrosis factor-α (TNFα)
vascular endothelial growth factor-A (VEGF-A)
vascular endothelial growth factor-A (VEGF-A) receptor-2 (VEGFR-2)

Introduction

Critical limb-threatening ischemia (CLTI) is a clinical syndrome defined by the presence of peripheral artery disease (PAD) in combination with rest pain, gangrene, or lower limb ulceration with more than 2 weeks of duration.[1] The condition is associated with significant morbidity, mortality, and increased utilization of health care resources.[2] The risk of major amputation due to CLTI is higher in patients with diabetes mellitus (DM) than CLTI patients without DM.[3, 4] Diabetes was highly prevalent in recently conducted randomized controlled trials in CLTI patients, such as the JUVENTAS (Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation) trial.[5] A sub-analysis of the JUVENTAS trial confirmed that, among patients with DM, the risk of amputation is higher in those with more severe ischemia at initial presentation.[6]

In this review article, we critically analyse the available evidence on the participation of the bone marrow (BM) in the pathogenesis of advanced vasculopathy in patients with diabetes. We report seminal data showing BM-derived circulating cells have proangiogenic properties that are lost in people with diabetes. Next, we refer to studies pinpointing a new pathologic phenomenon, for which the term mobilopathy was coined. This consists of the deficient liberation of BM cells as a result of a disrupted chemokine gradient within the BM and between the BM and the circulation. Importantly, the recognition of mobilopathy has fuelled the formulation of new predictors of adverse cardiovascular outcomes in people of DM. Finally, we report new findings highlighting that the BM of people with DM is affected by typical complications such as microangiopathy and neuropathy, which profoundly disturb the correct function of the marrow niche. Stem cells (SCs) and progenitor cells (PCs) residing in this harsh environment will be primed to dangerous phenotypes

transmissible to the peripheral microvasculature. Therefore, we believe that, as highlighted in the title of this review, the BM is target and accomplice of vascular complications in DM.

BM structural organization

The BM is the major reservoir for hematopoietic stem/progenitor cells (HSPCs). The marrow environment is not homogeneous. It comprises specialized microenvironments (niches) that correspond to stages of HSPC maturation, under the influence of an orchestrated network of soluble signals and surface interactions.[7] HSPCs characterized by high self-renewal capacity are localized near the endosteum and move to the bone marrow sinusoids as they mature. Endosteal stromal cells, endothelial cells of vascular sinusoids, and adventitial perisinusoidal cells support hematopoiesis through paracrine signalling and physical interactions. Moreover, BM vascular cells preside the regular trafficking of HSPCs to the systemic circulation and back to the BM. Importantly, both niches are highly innervated. Sympathetic innervation regulates both the acute and circadian release of HSPCs.[8] Moreover, this neuronal mechanism is responsive to pharmacological modulation, as for granulocyte-colony stimulating factor (G-CSF) whose administration induces HSPC mobilization, at least in part through indirect activation of noradrenergic receptors.[9]

The detrimental effect of DM on BM-derived angiogenic cells

The first demonstration of a damaging effect of DM on BM-derived cells was obtained on ill-defined cell populations, thought to be direct precursors of endothelial cells and endowed of proangiogenic activity in culture; therefore, named endothelial progenitor cells (EPCs).[10] EPCs were originally described in the late 1990s as BM-derived

circulating immature cells expressing the surface marker CD34 in combination with the vascular endothelial growth factor-A (VEGF-A) receptor-2 (VEGFR-2).[11] This pioneering paper inspired the new paradigm that hematopoiesis and angiopoiesis were mutually interactive phenomena and that the BM may play fundamental roles in vascular regenerative processes. The prevailing view was that EPCs generated in the BM could be mobilized following a gradient of growth factors and chemokines released by ischemic or injured tissues, reach the damaged tissue, and differentiate into endothelial cells. It is now clear that EPCs are not true progenitor cells, but belong to the category of myeloid cells, which support vascular growth mainly by secreting proangiogenic factors. Likewise, mesenchymal stromal cells and HSPCs subpopulations, including those expressing the surface marker CD34, can exert proangiogenic activities *via* paracrine mechanisms.[12, 13]

Diabetic mobilopathy

As mentioned above, there is regular physiological trafficking of HSPCs and other BM cells to the circulation. These patrolling cells are thought to participate in the homeostasis of peripheral vasculature and in sensing environmental signals that require BM reactions. A much more robust and forced mobilization occurs in response to peripheral injury and is governed by changes in the local concentration of chemokines. For instance, the CXC chemokine CXCL12 plays key roles in hematopoiesis, angiogenesis and inflammation. These actions are mediated by the binding of CXCL12 to the cognate CXCR4 and CXCR7 receptors on HSPCs and leukocytes.[14] High BM levels of CXCL12 retain those cells in the endosteal environment, whereas an inversion of the CXCL12 gradient results in cell mobilization into the circulation. Cytokines, such as G-CSF or granulocyte–macrophage colony-

stimulating factor (GM-CSF), are used to mobilize HSPCs into the circulation and harvest them for therapies in cancer patients. G-CSF induces the mobilization of HSPCs through its direct effects on macrophages and β-adrenergic sympathetic nerves, resulting in the dynamic modulation of the CXCL12 gradient between the endosteal and vascular niche.

Ferraro et al. were the first to report that DM alters the ability of sympathetic nerves to mediate the G-CSF–induced HSPC mobilization.[15] Another fundamental study in a large population with coronary artery disease showed that reduced circulating levels of HSPCs, identified for the expression of the cell surface marker CD34, are associated with risk of death in people with DM.[16] A definitive meta-analysis of 21 studies, comprising 4,155 individuals, confirmed this association.[17] Together with the notion that CD34+ cells maintain cardiovascular health, these studies suggest that an impaired liberation of reparative proangiogenic cells from BM to the circulation may be implicated in the increased cardiovascular vulnerability of diabetic patients.[18] Other conditions and risk factors, such as arterial hypertension and smoking, are characterized by a defect in HSPC mobilization,[19, 20] but, for none of them, such a strict association with risk of death has been reported.

Molecular mechanisms of mobilopathy

Mechanistic studies indicate that DM contributes in impairing BM cell mobilization *via* dysregulation of the key lifespan determinant pathway comprising the silent information regulator (SIR)T1, p66Shc, and mammalian target of rapamycin (TOR).[21-23] These genes integrate longevity pathways and metabolic signals in a complex interplay in which lifespan appears to be strictly dependent on substrate and energy bioavailability.[24] Recent data from Fadini's group suggested that genetic and

epigenetic changes in the aforementioned signalling pathways can occur as a consequence of DM in HSPCs, leading to combined detrimental effects on myelopoiesis and cell mobilization. This new mechanistic understanding could inspire novel modalities to restore BM regenerative capacity and preserve proper myelopoiesis in people with DM.[25]

Pathogenic phenomena upstream of mobilopathy

In the last 10 years, our team has been exploring the hypothesis that DM-related peripheral complications, namely microangiopathy and neuropathy, could extend to the BM and thus contribute to altering HSPC viability and functionality.

Initially, we demonstrated the presence of microangiopathy in BM of type 1 diabetic mice.[26] Rarefaction of capillaries and arterioles was associated with a depletion of HSPCs in the endosteal niche. This cell depletion was accompanied by increased oxidative stress, DNA damage, and activation of apoptosis. BM microangiopathy could be rescued by benfotiamine, which acts as a cofactor of transketolase thereby promoting the activity of the pentose phosphate pathway and reducing the accumulation of toxic metabolites of glycolysis.[26]

We next confirmed the occurrence of microangiopathy in the BM of patients with type 2 DM and verified this was more pronounced in those with CLTI.[27] To dissect the worsening effect of CTLI on the DM-induced BM damage, we compared BM samples from cohorts of patients (with or without DM) undergoing hip replacement surgery with those from CTLI patients requiring over-the-knee amputation. In the latter case, the BM was collected at the amputation level where viable tissue was still present. Analysis of microvascular density demonstrated a significant difference among the three groups for all sets of vascular structures considered, capillaries,

arterioles and sinusoids. There was a large decrease in vascular density in BM of patients with DM and this deficit was more pronounced in those with CTLI. We concluded that DM causes microangiopathy in human BM, with vascular rarefaction being aggravated by CLTI. In a multivariate analysis considering associated risk factors, microvascular rarefaction was predicted by the duration of DM and arterial hypertension, thus suggesting an interaction between the two conditions.[27] Further investigation of different populations of vascular and perivascular cells (pericytes) revealed that both carry alterations in key molecular signalling pathways that control angiogenesis, permeability, and leukocyte trafficking.[28, 29] Moreover, the rarefaction of microvessels was associated with apoptotic pauperization of $CD34^+$ HSPCs and fat accumulation.[27] The depletion of HSPCs was attributed to the creation of a hypoxic environment causing the downregulation of signallings that control the viability and proliferation/expansion of HSPCs.[27]

Furthermore, in line with concept that perception of pain is often detrimentally abrogated in diabetic patients with neuropathy, we found substance-P (SP) expressing nociceptive fibres were also reduced in the BM of patients and mice with type 1 and 2 DM.[22 30] These preclinical studies provided experimental evidence suggesting that BM sensory neuropathy might contribute in impairing the mobilization of specific populations of HSPCs. In diabetic mice with acutely induced hindlimb ischemia, we could observe that spontaneous blood flow recovery was delayed compared with non-diabetic mice. This expected difference was mirrored in diabetic mice by an altered gradient for SP between BM and the circulation and a reduced peripheral recruitment of populations of HSPCs responsive to SP.[22 30] This adverse post-ischemic outcome was phenocopied in mice that had the BM transplanted with HSPCs lacking receptors for SP and thus unable to respond to SP endogenously produced by sensory nerves.[22]

Importantly, both sensory nerve rarefaction and ischemic limb healing were rescued by gene therapy with nerve growth factor (NGF) in diabetic mice.[30] We concluded that, in patients with advanced diabetic complications, microangiopathy and sensory neuropathy could cooperate in jeopardizing HSPCs fate and function within the BM before they are liberated into the circulation. A recent review article has summarized the knowledge on neuropathy and inflammation in diabetic bone marrow.[31]

DM drives stromal cells away from bone toward an adipogenic fate

Patients with DM suffer problems with bones, including osteoporosis, more frequent fractures, and Charcot foot. The role of osteoblasts in DM-related osteoporosis is well acknowledged whereas the role of osteoclasts (OCLs) is still unclear. We found that type 1 diabetic mice have an increased number of active OCLs in trabecular bone. This was induced by the development of local hypoxia-induced acidosis in BM with consequent stimulation of transient receptor potential cation channel subfamily V member 1 (TRPV1).[32] This receptor is expressed by various cell types, including neurons. Interestingly, treatment with TRPV1 receptor antagonists restored OCL activity and bone homeostasis. We concluded that the use of clinically available TRPV1 antagonists may provide a new means to combat bone problems associated with DM. [32]

Stromal cells resident in the BM have the capacity to develop into bone and adipose tissue and this balance is maintained through a finely tuned mechanism controlled by genetic and epigenetic factors. We recently reported that type 2 DM causes molecular and metabolic changes that relentlessly convert the marrow stroma into inflamed fat.[33] The BM of people with DM, especially those affected by CLTI, becomes replenished with large adipocytes that compress the hematopoietic tissue

and secrete large amounts of chemokines and adipokines, such as tumour-necrosis factor-α (TNFα) and monocyte chemoattractant protein-1/chemokine (C–C motif) ligand-2 (MCP-1/CCL2), promoting macrophage infiltration.[33] CCL2 levels were also increased in the cell-free the BM lavage, suggesting that the chemokine could be released into the circulation.[34] Moreover, we discovered a feedback loop wherein BM stromal cells from diabetic patients are more inclined to make adipocytes and, in turn, mature adipocytes fuel stromal cell differentiation into new fat cells *via* CCL2 (**Figure 1**).[34]

To verify the contribution of CCL2 to BM adipogenesis, we administered a CCR2 antagonist or its vehicle to obese diabetic mice. Importantly, antagonist-treated mice showed less BM fat, due to reduced adipocyte quantities and size and also resulted in improved control of the diabetic condition.[34]

Involvement of BM HSPC dysfunction

Prediction of clinical outcomes in diabetic patients suffering CLTI is unsatisfactory. We performed a prospective study investigating if the abundance and migratory activity of circulating CD34+ HSPCs can predict major amputation and cardiovascular death in type 2 diabetic patients undergoing percutaneous transluminal angioplasty for CLTI.[35] Results of the study were unexpected and intriguing. At 18-month follow-up, time-to-event analysis of amputation and cardiovascular deaths showed no association with the quantity of the predictor. However, the ability of cells to migrate toward CXCL12 *in vitro* was strongly associated with cardiovascular death compared with event-free subjects. Multivariable regression model analysis showed that cell migration forecasts cardiovascular mortality independently of other validated predictors, such as age, diagnosed coronary artery disease, serum C-reactive protein, and estimated

glomerular filtration rate. In this model, the doubling of migrated cell counts increases the cardiovascular death hazard by 100%. We have confirmed the results in a follow-up analysis at 6 years. We concluded that the new predictor could aid in the identification of high-risk patients with type 2 diabetes requiring special therapeutic care after revascularization. These data suggest an association between increased cell migration and cardiovascular mortality. However, the observed association was not enough to establish a cause-effect relationship. We further explored the hypothesis that DM could convert HSPCs into damaging cells, thereby amplifying the direct injury of DM on the vasculature. Results clearly demonstrated that, in HSPCs, vascular cells and peripheral blood of patients with DM and CLTI, several anti-angiogenic microRNAs, such as microRNA-15a and microRNA-16, were increased; whereas microRNAs with proangiogenic activity were reduced. [36, 37] Furthermore, the anti-angiogenic microRNAs could be secreted and transferred to vascular cells thereby activating proapoptotic signalling in targeted cells. It is, therefore, possible that anti-angiogenic CD34+ HSPCs able to migrate to areas of ongoing vascular damage can further accelerate the pathological process and increase the risk of cardiovascular death (**Figure 2**).

Conclusions

Optimal management of the diabetic foot ulceration involves a multimodality approach directed at regular foot care, early recognition, restoration of perfusion in case of ischemia and metabolic control. However, a "*foot-centric*" approach may be short-sighted when considering the complex pathogenic phenomena occurring at a systemic level. This review highlights the necessity of considering the BM as a previously unforeseen contributor and a target of diabetic complications. Prevention of BM

deterioration could aid the treatment and care of the diabetic foot and the patient as a global entity.

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Figure 1: DM forces stromal cells to an adipogenic fate in BM. The cartoon illustrates the proposed transition from normal to inflamed BM adipocytes (ADs), with triglyceride (TG) accumulation, activation of CCL2 signalling to enhance stromal cells conversion into new enlarged ADs and recruitment of macrophages. Damaged ADs release proinflammatory factors, TGs and free fatty acids (FFA) that can be captured by cardiovascular tissue.

Figure 2. A vicious cycle affecting reparative mechanisms in DM. Microangiopathy and sensory neuropathy alter the BM niche, causing depletion and dysfunction of HSPCs and adipogenic transformation of stromal cells. Diabetic mobilopathy is associated with the release of cells that cause additional damage through secretion of anti-angiogenic and pro-apoptotic factors that are captured by the vascular endothelium. This can amplify the direct damage of metabolic disease on peripheral vessels. Inflammatory mediators from injured tissue (e.g. a diabetic foot ulcer) can reach the BM as well as other organs perpetuating a chronic low-grade systemic inflammation. Following angioplasty of CLTI, the presence of CD34+ cells characterized by high migratory activity identifies patients at high risk of cardiovascular mortality. The cause-effect relationship of this association remains undetermined, but it is possible that these cells contribute to persistent vascular damage.