

Endosteal vessel integrity: a new therapeutic goal in acute myeloid leukemia?

Paolo Bernasconi, Oscar Borsani

Division of Hematology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy

Correspondence to: Prof. Paolo Bernasconi. Divisione di Ematologia, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Piazzale Golgi 19, 27100 Pavia, Italy. Email: p.bernasconi@smatteo.pv.it.

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In acute myeloid leukemia (AML) the cure rate of elderly and some molecularly normal patients below 60 years of age is still 5–15% and about 60–80% patients still die due to chemo-resistance. Thus, in AML there is an urgent need to develop more effective treatments that should target not only the AML cell but also the specialized bone marrow (BM) microenvironment (“the niche”) whose critical role has been progressively defined (1-3). At first BM microenvironment was separated into endosteal and vascular niche, later differentiated in the arteriolar niche which contains NG2⁺, LepR⁻ CXCL12-abundant reticular (CAR) cells and one-third of HSCs and sinusoidal niche which carries NG2⁻, LepR⁺ CAR cells and two thirds of HSCs. The Sca-1⁺, VEGFR2⁺ arterioles with their CAR cells maintain HSCs quiescent, whereas the sinusoidal CAR cells retain and allow HSC expansion. Nowadays, these niches are no more considered distinct entities, but rather tightly intertwined dynamic structures that present a tight crosstalk with the HSCs (4). This finding and the biological and phenotypic similarities between HSCs and leukemia stem cells (LSCs) have founded the assumption that a strict dialogue between LSCs and the niche might also occur in AML. Despite the complexity of this interaction and some confusing information, various seminal studies have highlighted the molecular signaling pathways of this dialogue and suggested that in the near future these pathways may become novel potential therapeutic targets. The recent article published in *Cell Stem Cell* by a research group lead by Duarte *et al.* (5) represents a novel and relevant step in this direction since it identifies the specific endosteal vessel loss associated with AML as a new potential

therapeutic target. In the past AML engraftment was reported to occur around vessels of a specialized E-selectin and CXCL12 positive endothelium, but subsequently it was reported that AML engraftment occurs in BM areas enriched with osteoblasts. Recent studies showed that LSCs may remodel the niche by altering the localization of CD34⁺ cells through cytokine production and stimulating the creation of walled-off abnormal niches (6), while the niche may mitigate the effects of cytotoxic chemotherapy (7). Current studies have gone one step further by demonstrating that the interactions between the malignant cells and the BM microenvironment may vary in relation to disease subtype and stage (5). The only disease that challenges this concept is T-ALL whose highly motile and exploratory cells are stochastically distributed within the BM space and do not depend on specific niches for propagation and chemo-resistance (8). In AML a disease subtype specific relationship has been revealed by the demonstration that the propagation of MLL-AF9-driven AML was promoted by osteoblastic expansion that instead halted the expansion of BCR-ABL CML-like disease (9). Furthermore, the existence of a disease stage specific interaction was supported by the finding that the niche was necessary for leukemogenesis in a preleukemic stage, became permissive once leukemia has been established (1), and was transformed into a self-reinforcing leukemic niche at a later disease stage (2). The Duarte’s study (5) supports these concepts by revealing an association between unrelated AML types and a specific endosteal vessel loss that occurs as a primary AML-induced niche alteration probably responsible of the severe and life-threatening

cytopenias of these patients. The fundamental role of this loss in AML pathogenesis is expected since the vascular barrier is essential for oxygen, nutrients and drug delivery and its alteration is anticipated to cause increased vascular permeability, hypoxia, altered perfusion and reduced drug delivery. It has been previously reported that AML patients and murine models present an increased number of sinusoidal vessels within the BM central region. This alteration, termed BM “microvessel density” (BM-MVD) is still used to quantitate the central marrow vascularity and is not only promoted by MSCs and megakaryocytes, but also induced by the AML cell itself. Leukemic cells may create autocrine and paracrine loops not only by producing vascular endothelial growth factor and angiopoietins 1 and 2, but also by expressing their respective receptors. Recently, the EC markers specific of the distinct BM vascular niches (10) were analyzed in mice with AML patients-derived xenografts (PDX) (11). This study reported a significant loss of ECs associated with sinusoids versus a significant increase of ECs associated with arterioles and many poorly perfused BM areas leading to a significant increase of BM hypoxia. At an early AML stage these last areas were localized in close proximity to AML cells, whereas at a later disease stage these areas were present within the entire BM space. In addition, AML engrafted areas appeared much more leaky than non-engrafted areas and the leukemic BM, in contrast to the normal BM which nestin-associated vessels present less permeability than Nestin⁻ vessels, showed an increased leakiness of both Nestin⁺ and Nestin⁻ blood vessels. A deeper insight on how ECs and blood vessels alterations evolve from early to late AML disease stage has been provided the Duarte’s study (5). First of all, vessel loss is a very specific event: it specifically and significantly involves the endosteum and the metaphysis and not the diaphysis. In addition, when BM infiltration is only 5–15%, perivascular and endosteal stroma are differently affected: they have a normal appearance in low infiltrated areas where blood vessels are narrower than those of control mice and far from the endosteal surface and are depleted in high infiltrated areas that present patches of AML cell clusters. At early disease stage blood vessels of high infiltrated areas and at late disease stage blood vessels of the entire BM displayed abnormal oscillations perhaps due to the loss of their anchorage to the surrounding parenchyma. Moreover, these vessels presented the sequential formation and retraction of sprouts that rendered the sprouting process never effective. Ultimately ECs collapsed into small debris that entered the vascular lumen

and could be taken up by AML cells, an event that may be the true mechanism through which AML cells produce angiogenic cytokines. In addition, the sequential formation and retraction of sprouts not only explains why the BM-MVD is still a useful even if not specific diagnostic marker in AML being increased on clinical diagnosis and normal on achievement of complete remission, but it also explains why anti-angiogenic factors have up to now provided modest results. Recently, a study has further strengthened the prognostic relevance of BM-MVD by demonstrating that the failure of induction chemotherapy to restore a normal BM-MVD is a very poor prognostic sign (11). In this study RNA sequencing of BM-derived ECs revealed an upregulation of integrin expression associated with the activation of the Fak pathway, a reduced expression of tight junction components and a high expression of the *Nox4* gene. This last encodes for a NAPDH oxidase expressed in the vasculature and involved in response to hypoxia via production of reactive oxygen species (ROS), activation of nitric oxide synthase 3 (NOS3) and release of nitric oxide (NO) (12). Instead, despite a similarity in gene expression pattern between endosteal and central AML cells, Duarte revealed that endosteal AML cells presented a significantly higher expression of inflammatory cytokines, especially tumor necrosis factor (TNF) and anti-angiogenic cytokine CXCL2. *In vitro* colony assays had already pointed out that TNF and adiponectin were responsible of the inhibition of hematopoietic progenitor cell growth induced by the BM interstitial fluid (IF) of AML patients as antibodies against these cytokines abolished this inhibition. Moreover, as the success of induction chemotherapy was anticipated by a reduction of these cytokine in BM IF, it was suggested that TNF and adiponectin may be used as AML prognostic markers. TNF was also included and over-expressed in the inflammation-associated gene signature that characterized the FLT3-ITD-induced disruption of HSC-supporting BM stromal cells, an alteration that lead to a significant and severe loss of long-term repopulating HSCs (13) and its inhibition partially rescued the HSC phenotype. Recently, TNF and CXCL2 were reported to promote endosteal vessel destruction (14) and HSC mobilization (15).

The other relevant information provided by Duarte (5) is that the AML-induced alterations of the microenvironment and normal hematopoiesis evolve focally and in parallel: a specific EC depletion occurs as a primary event and is followed by the concomitant loss of osteoblastic and HSC cells. The spatiotemporal order of these changes is confirmed by the observation that osteoblasts were

significantly decreased in areas with high levels of leukemia infiltration and maintained in areas of intermediate infiltration that showed a blood vessel loss only. A previous AML xenotransplantation model had already reported that an osteoblast depletion similar to that of MDS/AML reduced animal survival by causing leukemic cell propagation, enhanced BM and spleen leukemic engraftment and an alteration in lineage progression, events reverted by osteoblast maintenance (16). In contrast, another report suggested that differentiating osteoblasts may protect AML cells from CXCL12-induced apoptosis (17) and recently a pre-clinical breast cancer model has showed that marrows from mice treated with zoledronic acid (ZA), a potent bisphosphonate widely used as an osteoclast/osteoblast inhibitor, were metastasis-suppressive (18). All these studies did not establish when osteoblast depletion occurred, whereas the Duarte's study (5) clearly pinpoints the consecutive order of events that lead to the niche collapse. However, whether the EC and osteoblast cell loss were due to an alteration of Notch signaling, the pathway that mediates their tight molecular crosstalk was not investigated. Thus, it is not possible to evaluate whether the AML-induced alterations of the niche are similar to those observed in aged mice that present a defective Notch signaling leading to alterations of angiogenesis and osteogenesis reverted by bisphosphonate treatment (19). The finding that in young and aged mice this same treatment caused a significant increase of bone volume and HSC numbers (20) suggests that a tight crosstalk must also exist between osteoblasts and HSCs. This assumption is partially confirmed by the observations that modulations in the number of spindle-shaped N-Cadherin⁺ CD45⁻ osteoblasts cause an increase/decrease of HSC numbers and mature endosteal osteoblasts may reorganize the BM endosteal microenvironment after marrow ablation. Duarte (5) confirms the existence of this interaction by showing a concomitant loss of osteoblast and HSCs at a late AML stage. HSC loss involved both Lineage⁻, cKit⁺, Sca-1⁺ (LKS) progenitor cells and LKS CD48⁻, CD150⁺ HSCs. However, the former cells were depleted from areas both distant and close to the bone, while the second cell subset was dramatically depleted from bone-rich metaphysis. This observation contrasts with the reported impairment of normal HSC differentiation at the level of HSC-progenitor cell transition (21) and the progressive HSC quiescence determined by the AML-induced down-regulation of cell-cycle genes and up-regulation of several other genes including *Hes-1* and *Egr3* (22). The HSC

depletion reported by the Duarte's study (5) nourishes the idea of a competition between LSC and normal HSCs with a direct AML-induced niche remodeling leading to a self-reinforcing malignant structure able to support and strengthen the AML population with respect to normal hematopoiesis (3). This competition is further supported by the discovery that primary leukemia-initiating cells (L-ICs) may be consistently outperformed by normal cord blood (CB) CD34⁺ cells in a cell dose dependent manner (23) and higher doses of CD34⁺ cells are associated with a significant lower relapse incidence after allogeneic HSC transplant. In the Duarte's study (5) the BM loss of LKS and HSC cells was due to an increased leakiness of blood vessels, an event that another study suggested to be induced by the leukemic cell population that determined an increased expression of *NOX4* in ECs and an increased ROS production within the BM (11). This altered permeability lead to an increased HSC trafficking from the damaged BM blood vessels to the spleen, a situation reminiscent of primary myelofibrosis in which it was supposed that an imbalance between endosteal and vascular niches may occur. Interestingly, the concomitant increase of spleen ECs and LKS/HSC trafficking from the BM to the spleen supports the assumption that niche regulatory functions may re-initialize in an organ distinct from BM (i.e., "niche plasticity"), an event further sustained by the recent discovery of perisinusoidal spleen niches (24).

How this relevant information can be exploited to improve our patients' cure rate? Since at late disease stage the AML-induced niche collapse is no more reversible and prevents HSC homing, Duarte (5) suggests the suppression of EC loss as best therapeutic strategy to prevent the AML-induced endosteal vascular niche remodeling. Indeed, the integrity of endosteal and metaphyseal blood vessels may truly improve the AML cure rate by increasing the leukemic cell exposure to cytotoxic agents and avoiding LSC homing to BM hypoxic areas, the true "sanctuaries" of chemo-resistant cells (7). Thus, based on the fact that deferoxamine (DFO), an iron chelator often combined with differentiation therapy, induces endosteal vessel expansion by enhancing the stability and activity of hypoxia-inducible factor 1 α (HIF1 α), this study employed DFO to treat leukemic mice. Indeed, in these mice DFO rescued endosteal vessels and produced a significant increase of HSC numbers in the trabecular-rich metaphysis, but the number of BM AML cells, the survival and disease progression of DFO-treated were similar to those of control mice unless DFO was combined with chemotherapy. However, DFO not only

influences HIF1 α activity but also causes the differentiation of leukemic blasts and normal BM precursors into monocytes/macrophages by modulating ROS expression and activating mitogen-activated protein kinases (25) and acts synergistically with vitamin D3. Thus, the effectiveness of DFO reported by Duarte (5) might also rely on its ability to modulate BM ROS expression that may be at the crossroads of the endosteal vascular niche remodeling as hypothesized by another study (11). This last suggested that another potential therapeutic option might be to reduce the production of ROS and NO and the expression of NOS3 that were increased in the BM of PDX mice and AML patients due to the AML-induced upregulation of *Nox4* in ECs. In addition, this study underlined the poor prognostic significance of NO levels as in AML patients persistently elevated NO levels were associated with a 73% probability of treatment failure. Most importantly, in PDX mice NO inhibition combined with Ara-C reduced NOS3 activation, vascular leakiness, BM hypoxia and these mice presented a reduced AML progression in BM and spleen and a “remission-like” phase longer than control mice. In addition, this combined treatment by normalizing vascular permeability was more efficient than chemotherapy alone in re-establishing not only the number but also the function of BM SLAM⁺ cells allowing them to outcompete leukemic cells during the relapse process. The only drawback of NO inhibitors is that most of them act on NOS2 and very few on NOS3. Moreover, preclinical studies have not only clarified the mechanisms of NOS3 regulation and NO production but have also suggested that the best way to inhibit NO production would be to target its upstream regulators in ECs, a very difficult task as these regulators are many and act in a very complex way.

In conclusion, the deeper clarification of the spatiotemporal damage that AML cells induce within the niche at early and late disease stage is flagging the way for identifying new promising potential treatments that combined with standard protocol may enhance the efficacy of current strategies and patients' cure rate.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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