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Editorial

Role of the CLC-3 Channel Transporter in Angiogenesis by Endothelial Progenitor Cells in Ischemia-Induced Vascular Injury: A Key Passenger or a Driver?

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The past 2 decades have seen an explosion of new research exploring the potential of stem cell therapy to regenerate biologic tissues in various acute or chronic diseases or after accidental damage. Stem cells are recognized to have 2 main properties: 1) self-regeneration (or "self-renewal") in their undifferentiated state, and 2) potency, or the potential to differentiate into a specific cell type under specific stimulatory conditions.[1-3] Endothelial progenitor cells (EPCs) are similar to stem cells in that they share the ability to self-regenerate in an undifferentiated state (or "self-clone") and differentiate into true endothelial cells in response to stress that can then populate damaged blood vessels and generate new blood vessels or trigger angiogenesis from existing vessels. Their discovery in 1997 by Asahara et al.[4] reshaped our understanding on how new blood vessels are generated postnatally, which were originally thought to be formed exclusively via angiogenesis as opposed to vasculogenesis, which is the process of embryologic development of blood vessels from the mesoderm. Based on the expression profile of surface markers, their origin, cell culture conditions, and function, EPCs are now categorized into 2 general subclasses: early and late EPCs. Early EPCs, also called myeloid EPCs or myeloid angiogenic or proangiogenic cells, are circulating cells that originate from the bone marrow; among many others, they express CD-34, vascular endothelial growth factor receptor R (VEGF2, also referred to as kinase domain–containing receptor), CD-117, and CD-133. Late EPCs, also referred to as endothelial colony-forming cells, reside within the wall of blood vessels and express CD-34, CD-31, VEGFR2, von Willebrand factor, vascular endothelial cadherin (CD-144), and endothelial nitric oxide synthase.[1-3] Recent evidence suggests that early EPCs may respond to more severe damage to blood vessels (eg, following irradiation), whereas late EPCs seem to play an important role in all forms of blood vessel injuries occurring in normal physiologic (normal turnover of blood vessels) as well as pathophysiologic conditions. After mobilization from the bone marrow in response to hypoxia (via hypoxia-inducible factor 1 [HIF-1]), chemokines, growth factors, and others, early EPCs then migrate to the injured blood vessel wall and bind to adhesion extracellular matrix proteins such as selectins, integrins, fibrinogen, and fibronectin. After invading the impaired blood vessel wall, early EPCs then mediate their angiogenic activity through a paracrine mechanism involving the release of VEGF, HIF-1, and others, which stimulates differentiation of resident EPCs to remodel existing blood vessels or form new ones *de novo*.

In an elegant study published in the November 2018 issue of this journal, Cheng et al.[5] reported that CLC-3, a member of the CLC superfamily of Cl^- channels that is ubiquitously expressed in most if not all cell types,[6] plays a key role in the ability of early EPCs to promote neovascularisation in the hindlimb ischemia model in mice. The CLC family of anion transporters comprises 9 members in mammals. Of these 9 members, 4 are considered to be true anion-permeating ion channels: CLC-1, CLC-2, CLC-Ka, and CLC-Kb. In contrast the remaining 5 members, CLC-3 through CLC-7, are now considered to be electrogenic secondary antiporters, or exchangers that transport 2 Cl^- ions across the membrane in exchange for 1 proton ($2Cl^-/H^+$ exchanger) per translocation cycle; it should be emphasized that the function of CLC-3 as a $2Cl^-/H^+$ exchanger has not yet been unequivocally confirmed. The exact subcellular localization of CLC-3 is still the subject of an intense debate with 2 radically different schools of thought. One school claims that the transporter is expressed at the plasma membrane of various cell types,[7-11] including endothelial cells.[12] Another and probably larger camp suggests that these transmembrane proteins are expressed in intracellular organelles, such as endosomes, lysosomes, and synaptic vesicles (as extensively reviewed by Jentsch and Pusch[6]). In the latter conceptual framework, $2Cl^-/H^+$ exchangers, in conjunction with an adenosine triphosphatase (ATPase)–mediated H^+ pump, is hypothesized to play an

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important role in ion homeostasis and acidification of organelles by shunting the accumulation of positive charges from H^+ pumped into the organelle by the H^+ pump ATPase.[6]

Using both *in vitro* and *in vivo* approaches as well as CLC-3 knockout (KO) mice, Cheng et al.[5] showed that EPCs derived from CLC-3 KO animals displayed impaired adhesion, migration, and angiogenic activity. Based on the expression of specific markers and the short time in cell culture (7 days), the authors concluded that the profile of the EPCs was consistent with those of early EPCs, but it is debatable whether the EPCs used by Cheng et al.[5] were in fact early, late, or an intermediate stage of differentiation because the expression of 1 key surface marker of early EPCs, CD-133 (and perhaps CD-117), was not probed by the authors. It is well accepted that this antigen is down-regulated during differentiation of early EPCs after invasion of an ischemic zone.[2,3] The authors mentioned that the early EPCs used were a mixed population of cells, which might have contained a subpopulation of monocytic cells capable of differentiating into late EPCs. It will be critical in the future to assess which of the early or late EPCs, or both, express CLC-3 and mediate their angiogenic effects with the aid of this anion transporter.

A key result of the Cheng et al.[5] study was the finding that CLC-3 KO mice displayed a reduced ability to restore blood flow after hindleg ischemia, as determined by quantitative Doppler flow measurements performed *in vivo*. Transplantation of EPCs from CLC-3 KO mice to wild-type mice similarly impaired the neovascularisation process involved in blood flow restoration during the recovery phase after hindleg ischemia. The authors also explored the role of the CXC chemokine receptor 4 (CXCR4) and its downstream target Janus kinase 2 (JAK-2) as potential mediators of neovascularisation in response to ischemia-induced injury and found that both proteins were down-regulated in EPCs in CLC-3 KO compared with wild-type mice. The negative impact of CLC-3 deficiency was shown to be overcome by overexpression of CXCR4 in EPCs from KO animals. Finally, and importantly, the authors also demonstrated that implanting EPCs from CLC-3 KO mice into wild-type mice mimicked the altered phenotype seen with CLC-3 KO mice, an observation in support of the argument that CLC-3 channels in EPCs play an important role in neovascularisation after ischemic injury.

One obvious question pertaining to this and other similar studies examining the role of CLC-3 in endothelial cell differentiation and proliferation is how this anion channel/antiporter mediates its effects on gene expression and ultimately function. As a plasma membrane anion channel, CLC-3 could mediate its effects by regulating membrane potential, transmembrane Cl^- fluxes, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and, indirectly, gene expression. As a transporter $2Cl^-/H^+$ exchanger protein located in endosomes (and possibly lysosomes), CLC-3 could likely influence protein trafficking, which could indirectly dictate gene expression.[6] In the study by Cheng et al.,[5] a link between CLC-3, CXCR4, and JAK-2 phosphorylation was clearly established. However, are these general effects of CLC-3 as an upstream regulator of multiple signal transduction pathways including CXCR4 and JAK-2, or is there a specific and unique link tying CLC-3 to CXCR4 and JAK-2 and leading to angiogenesis through changes in $[Ca^{2+}]_i$, vesicular protein trafficking, or a yet unidentified function of the protein? Future experiments should be devised to address these important questions to determine the role of CLC-3 not only in EPCs and neovascularisation, but also in other cell types in which this transporter has been speculated to participate in cell proliferation and differentiation.

Regardless of the controversy surrounding the exact subcellular distribution of CLC-3, its biophysical characteristics as a channel and/or transporter, and its functions, this transmembrane protein clearly plays a central role in physiologic and pathologic processes that regulate cell growth, including apoptosis, cell-cycle regulation, cell migration, cardiac and vascular functions in health and disease (eg, in acute myocardial ischemia, stroke, diabetes), and cancer development. In the rapidly expanding field exploring the feasibility of using EPCs to treat ischemic vascular diseases, tumours, and diabetes, the report by Cheng et al.[5] has potentially identified a new signalling target for diagnosis and therapeutic intervention.

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Disclosures

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