Deficient CDKN2B Expression
A Double Hit for PAD

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In 2007, multiple independent groups, using genome-wide association studies, made a significant breakthrough in the field of cardiovascular genetics when they identified a coronary artery disease risk locus on chromosome 9p21. Subsequently, additional studies examining a wide variety of patient cohorts have linked single-nucleotide polymorphisms (SNPs) within chromosome 9p21 to several cardiovascular diseases, including myocardial infarction, stroke, aneurysms, and peripheral artery disease (PAD).\(^1\) Importantly and perhaps unexpectedly, genetic variation at chromosome 9p21 has been shown to modify PAD risk independent of conventional atherosclerotic risk factors or pre-existent myocardial infarction.\(^2,3\) Although rates of coronary artery disease and first myocardial infarction may be decreasing,\(^4\) PAD is showing no evidence of a decline, and with roughly 1/5 of the population carrying 2 copies of the 9p21 risk allele,\(^5\) a role for this locus in PAD pathogenesis is intriguing.

The authors present exciting evidence for a role of CDKN2B in PAD pathogenesis.

The authors first report the striking observation that human coronary atherosclerotic plaques from carriers of the 9p21 risk allele have increased intraplaque microvessel density but impaired \(\alpha\)-smooth muscle actin coverage of these microvessels. Increased microvessel density within atherosclerotic lesions has been linked to increased rates of intraplaque inflammation, hemorrhage, and rupture.\(^6\) This human observation by Nanda et al\(^6\) suggests that it may not only be the presence of endothelial cells (ECs) within the plaque that is important but also whether the ECs are invested by perivascular cells, be it SMCs or pericytes. Throughout the study, the authors use \(\alpha\)-smooth muscle actin staining, which cannot distinguish between SMCs and pericytes, as pericytes can also express \(\alpha\)-smooth muscle actin, particularly in an inflammatory setting.\(^7\) In fact, distinguishing between SMCs and pericytes, at least in mouse, requires rigorous lineage tracing, use of multiple markers, and high-resolution confocal microscopy.\(^8\) Regardless, whether it is paracrine effects from ECs, the higher rates of EC growth, or defects within the perivascular cells themselves that lead to an increased number of immature blood vessels is an important area of investigation and potential therapeutic modulation. The authors’ initial observation linking the 9p21 risk allele to increased microvessel density, coupled with their previous discovery that the 9p21 risk allele correlates with reduced CDKN2B expression in atherosclerotic plaques,\(^6\) together suggest a possible role for CDKN2B in atherosclerotic plaque progression and thus PAD.

The authors proceed to ask whether the perivascular cell coverage defect present in human atherosclerotic lesions can be recapitulated in a nonatherosclerotic setting and use hindlimb ischemia (HLI), a well-established mouse model of PAD, to answer this question. On induction of HLI in global CDKN2B\(^{-/-}\) mice, they see decreased \(\alpha\)-smooth muscle actin coverage of new blood vessels, which is accompanied by impaired blood flow recovery and increased hindlimb tissue loss. They also note decreased numbers of total microvessels in CDKN2B\(^{-/-}\) mice 2 weeks after HLI. Because the authors later show that ECs deficient in CDKN2B have a greater angiogenic capacity, this suggests that the relative loss of ECs may be because of the lack of a perivascular cell layer to stabilize them.

In well over 90% of patients, PAD is caused by atherosclerosis leading to occlusions of the large inflow vessels, and in a sizeable fraction of patients with symptomatic PAD, there is a total occlusion along the single path that blood takes from the aorta to the distal leg.\(^9\) As such, perfusion becomes entirely dependent on a patient’s ability to effectively remodel vasculature to permit distal tissue perfusion. In many ways, much like the response to HLI in mice is under genetic control, the
response to the total occlusion in inflow (iliac or femoral) arteries is highly variable from patient to patient. The greater the neovascular response, the less the patient is afflicted. It is interesting to note that roughly half of patients with PAD, despite having a reduced ankle-brachial index, report no symptoms, suggesting their ability to mount an effective neovascular response. In light of the findings by Nanda et al., could reduced CDKN2B expression lead to impaired functional neovascularization and therefore worse outcomes for patients with PAD? Conversely, if an iliac or proximal femoral artery occlusion occurs in a patient, can robust CDKN2B expression allow sufficient revascularization so that the PAD becomes undiagnosed? If so, reduced CDKN2B expression, by both accelerating atherosclerotic disease progression and impairing an effective neovascular response, may be a “double hit” for PAD.

The authors next turn to in vitro studies to determine how reduced CDKN2B expression leads to impaired neovessel maturation. Using siRNA-mediated knockdown of CDKN2B in individually cultured ECs and SMCs exposed to hypoxic (2% oxygen) conditions, they find that ECs have increased angiogenic properties, including increased migration and proliferation. Of note, these effects are severely reduced in the absence of hypoxia. This adds to other data suggesting that hypoxic endothelium in hindlimb muscle may well respond differently than ECs that are not under hypoxic stress. In vascular SMCs, CDKN2B knockdown has minimal effects on migration and proliferation when grown under hypoxia. When they coinject CDKN2B-deficient ECs and SMCs using a matrigel plug assay, they are able to recapitulate the phenotype observed in vivo, that is, poor SMC coverage of endothelium.

This study is now the third by the Leeper group to use CDKN2B−/− mice and demonstrate a unique SMC phenotype. Although aneurysm and atherosclerotic plaque development are often associated with defective SMC function, the role of the SMC in neovessel formation/angiogenesis is clearly understudied. The fact that CDKN2B knockout leads to impaired SMC investment of new vessels, which correlates with impaired perfusion recovery and increased tissue loss, highlights the importance of SMCs to functional blood flow. SMCs, after all, must detach from the vessel wall, proliferate, migrate, and ultimately reattach to form new functional vessels capable of blood flow. The finding that global CDKN2B−/− seems to positively affect ECs (proangiogenic effects) but negatively affect SMCs (poor SMC coverage of vessels) and yet still lead to a net negative overall phenotype is further support for the essential role of perivascular cells to functional blood flow. In the future, it may be interesting to use cell-specific knockout of CDKN2B to tease out the relative contributions of ECs versus SMCs to the observed phenotype.

The fact that Nanda et al. have uncovered a novel mechanism whereby reduced CDKN2B expression affects both atherosclerotic and nonatherosclerotic diseases, via inhibition of neovessel maturation, is exciting, particularly in light of recent genome-wide association studies. Several studies have linked SNPs within the 9p21 locus with PAD independent of atherosclerotic risk factors. Could this be the mechanism behind it? Although the data are encouraging, the story is likely much more complicated than a single SNP in a single gene leading to various cardiovascular diseases. Recall that another cell cycle inhibitor, CDKN2A, and its splice variant p14/ARF are also located just proximal to the 9p21 haplotype block. Mouse models using knockout of this complex have been linked to accelerated atherosclerosis. Another gene with exons overlapping the INK4/ARF locus, methylthioadenosine phosphorylase, has also been linked with SMC proliferation and apoptosis. In addition, the 9p21 locus itself contains a long intergenic noncoding RNA, termed antisense noncoding RNA in the INK4 locus. Antisense noncoding RNA in the INK4 locus is capable of recruiting transcriptional repressive complexes to epigenetically repress various loci, including the CDKN2B promoter. Taken together, it is possible that each of these genes may contribute to disease risk independently or through combinatorial mechanisms.

Finally, the authors use a series of cDNA microarrays to identify ≈250 genes significantly dysregulated in CDKN2B-deficient ECs and SMCs. Pathway analysis shows that the majority of dysregulated processes involve angiogenesis or transforming growth factor-β (TGF-β) signaling. To confirm the involvement of the TGF-β signaling pathway, they analyze human carotid endarterectomy samples and show an inverse correlation between CDKN2B and TGF-β expression. They use a series of ELISAs, polymerase chain reaction, and Western blots to further explore how the TGF-β signaling pathway is altered under hypoxic conditions in the setting of reduced CDKN2B expression. Hypoxic ECs and SMCs have decreased expression of the inhibitory factor SMAD7, upregulation of TGF-β1, increased SMAD3 activation, and ultimately upregulation of the focal adhesion molecule TGF-β11. Then, they return to the EC–SMC matrigel plug assay and demonstrate that simultaneous siRNA-mediated inhibition of both TGF-β11 and CDKN2B leads to a normalization of vessel maturation, that is, the phenotype can be rescued in an in vitro setting through modulation of the TGF-β pathway.

PAD is a growing public health problem for which no medical therapies exist that are effective in improving perfusion to the lower extremities. Could modulation of TGF-β signaling be a potential therapeutic target to effectively improve perfusion by promoting neovascularization? It is probably not so simple, as TGF-β signaling is responsible for a wide range of cell and context-dependent effects. For example, TGF-β signaling has been shown to have proangiogenic or antiangiogenic effects on ECs depending on whether signaling occurs through ALK1 and SMAD1/5 or through ALK5 and SMAD2/3, respectively. The story is similar in vascular SMCs, where TGF-β1 can either promote the contractile state through myocardin/serum response factor interactions at CArG boxes or the synthetic state via effects on proliferation and extracellular matrix synthesis.

In this study, the authors also note cell-dependent effects, which may complicate future therapeutic strategies. In addition, although only ECs and SMCs were studied, TGF-β signaling can also affect function and viability of skeletal muscle, an often overlooked component of an effective neovascular response after HLI. As a downstream effector molecule, TGF-β11 may prove to be a more promising therapeutic target. In the future, it will be informative to see whether increased TGF-β1 or TGF-β11...
expression correlates with the 9p21 risk allele and, in turn, reduced CDKN2B expression in other human tissue samples.

In summary, this study provides a novel mechanism linking the 9p21 risk allele with reduced CDKN2B expression, increased TGF-β signaling, and impaired neovessel maturation. Importantly, in line with previous genome-wide association study reports, these correlations seem to be present under atherosclerotic and nonatherosclerotic conditions, suggesting that CDKN2B may both promote atherosclerosis progression and impair functional neovascularization, effectively a “double hit” for PAD pathogenesis.

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References

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