Role of microRNAs in peripheral artery disease (Review)

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Abstract. Peripheral arterial disease (PAD) involves a general vascular problem of diffuse atherosclerosis. The key pathological process is characterized by the aberrant proliferation of vascular smooth muscle cells and the formation of neointimal lesions. The molecular mechanisms involved in the regulation of the occurrence and development of PAD remain unclear. microRNAs (miRNAs) are highly conserved 20-25 nt-long non-coding RNAs that negatively regulate gene expression. Recent evidence has demonstrated that specific miRNAs are involved in the pathological processes of PAD, and these miRNAs are found to be critical modulators of vascular cell functions, including cell differentiation, contraction, migration, proliferation and apoptosis. This review summarizes findings of studies regarding the roles of specific miRNAs in PAD.

Contents

1. Introduction

Peripheral arterial disease (PAD) affects 12-14% of the general population, and its prevalence increases significantly with age. PAD is most frequently caused by atherosclerosis (AS), which leads to obstruction of the blood flow in the lower extremities. The most significant risk factors for the development of PAD are diabetes mellitus (DM) and tobacco abuse, although hypertension and hyperlipidemia are also contributing factors (1,2). Despite progress towards the prevention and treatment of PAD, understanding of the pathogenesis remains only at the initial stage. Understanding the cellular and molecular mechanisms that lead to the development of PAD is critical for identifying strategies to limit disease progression before it has clinical consequences.

Recent studies (3) have paid increasing attention to the cellular and molecular mechanisms of PAD, in which microRNAs (miRNAs) have been the focus. miRNAs are an emerging class of highly conserved, non-coding small RNAs (20-25 nucleotides) that regulate gene expression at the post-transcriptional level, inhibiting the translation of protein from messenger RNA (mRNA) by promoting the degradation of mRNA. miRNAs are key regulators of numerous events, including the balance between cell proliferation and differentiation during tumorigenesis and organ development. At present, more than 600 human miRNAs have been identified, and it has been proposed that they regulate over 50% of human protein-coding genes (4,5). Significantly, one miRNA is able to regulate the expression of multiple genes due to its ability to bind to its mRNA targets as either an imperfect or perfect complement. Thus, one miRNA is as functionally important as a transcription factor. As a group, miRNAs may directly regulate at least 30% of the genes in a cell and therefore are involved in the regulation of all major functions (6).

Over the last few years, there has been an increase in miRNA research due to the identified significant roles of these non-coding RNAs in cardiovascular pathophysiology, including arteriosclerosis, angiogenesis and intima hyperplasia. Basic and clinical studies have suggested that miRNAs are important regulators of vascular cell differentiation, growth, proliferation and apoptosis (7-9). However, their biological roles in PAD have only been elucidated recently. Several studies, including our research group, have demonstrated that miRNAs are important in PAD and PAD-associated complications. In the present review, basic and clinical research regarding miRNAs in PAD is summarized and a perspective for this new frontier of peripheral arterial ischemic disease research is provided.

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2. Endothelial cells and miRNAs

It is estimated that an adult human has approximately 1-10x10^{12} endothelial cells (ECs) lining the inside of blood vessels. Among these cells, only approximately 0.01% are estimated to be present in the cell cycle at any given time (10). ECs are major regulators of vascular health. During certain physiological events, including wound healing and oxidative stress, alterations in hemodynamic forces or pathological stimuli, such as inflammation or AS, cause ECs to assume a phenotype that predisposes to proatherogenic alterations (11). Therefore, evaluation of endothelial functions may provide insights into the mechanisms underlying the initiation, progression and clinical manifestations of PAD. A number of previous studies have verified the particular role of miRNAs in regulating the function of vascular ECs.

Human umbilical vein endothelial cells (HUVECs) are a valuable *in vitro* model of angiogenesis due to their ability to form capillary-like structures in response to appropriate stimuli (12). The miRNA signature of HUVECs has been recently determined, and several highly expressed miRNAs have been found to have angiogenic growth factor receptors as their putative targets (13). Among these, the following miRNAs play important roles.

miR-21. miR-21, a negative modulator of angiogenesis, has been demonstrated to be highly expressed in ECs. A study by Sabatel *et al* demonstrated that miR-21 overexpression reduced EC proliferation, migration and the ability of these cells to form tubes, while the inhibiton of miR-21 using a LNA-anti-miR led to opposite effects (14). Other studies (15) have demonstrated that the overexpression of miR-21 also led to a reduction in the organization of actin into stress fibers, which may explain the decrease in cell migration. Results from another study demonstrated that miRNA-21 exhibits an antiangiogenic function by targeting RhoB expression in ECs (16). Findings of other studies have also suggested that mechanical forces associated with blood flow play a role in regulating vascular signaling and gene expression in ECs. miR-21 therefore affects these pathological processes by decreasing apoptosis and activating the nitric oxide (NO) pathway (14). Adhesion of circulating monocytes to vascular ECs is a critical event leading to vascular inflammation and development of AS. In this pathological process, miRNA-21 targets the peroxisome proliferator-activated receptor α (PPARα) in an autoregulatory loop to modulate flow-induced endothelial inflammation (17).

miR-126. Adhesion molecules expressed by activated ECs are important in the regulation of leukocyte trafficking to sites of inflammation. Findings by Harris *et al* (16) demonstrated that the overexpression of miRNAs in EC inhibits vascular cell adhesion molecular 1 expression, which leads to inhibition of leukocyte adherence to ECs (18-20). miR-126 is also involved in cell apoptosis. miR-126 has been found to mediate CXCL12 production, which was enriched in apoptotic bodies, and induced vascular protection by repressing the function of the regulator of G protein signaling 16 (21). In addition, the beneficial role of miR-126 has been elucidated using the specificity of antagomir-induced silencing of miR-126 *in vivo* (22).

miR-221/222. Zhu *et al* found that miR-221/222 were highly expressed in HUVECs, and overexpression of the miR-221/222 targeted Ets-1, a key endothelial transcription factor of inflammation and tube formation, indirectly regulated the expression of several inflammatory molecules of ECs, which eventually attenuated cell adhesion and migration (23). In addition, miR-221/222 is involved in inflammation-mediated vascular remodeling by regulating the expression of the signal transducer and activator of transcription 5A (STAT5A) (24).

Other miRNAs are also involved in the regulation of ECs. miR-210 overexpression in normoxic EC cells stimulated the formation of capillary-like structures and vascular endothelial growth factor-driven cell migration (25). Oxidized low-density lipoprotein (ox-LDL) mediates EC apoptosis, which plays a critical role in AS. Through *in vivo* investigations examining the regulation of the expression of Bcl-2, miR-365 was confirmed to modulate ox-LDL-induced EC apoptosis (26). miR-424 promoted post-ischemic angiogenesis *in vitro* and in mice, which may regulate hypoxia-inducible factor 1α isoforms (27). Oscillatory shear stress (OS) induces the inflammatory response, a critical atherogenic event in which miR-663 has been identified to play a key role in OS-induced inflammatory responses by mediating the expression of the inflammatory gene network in HUVECs (28).

3. Vascular smooth muscle cells (VSMCs) and miRNAs

It is well-known that the phenotypic modulation of VSMCs from a differentiated phenotype to a dedifferentiated state, accompanied by accelerated VSMC proliferation, is crucial in the pathogenesis of a variety of proliferative vascular diseases, including AS, hypertension, restenosis after angioplasty or bypass and diabetic vascular complications, including PAD (29,30). Recent studies have established specific miRNAs as significant mediators of the modulation of VSMC phenotype by targeting transcription factors and the cytoskeleton, which act as molecular switches for VSMC differentiation (31).

miR-1. Myocardin is a cardiac- and smooth muscle-specific transcription co-factor that potently activates the expression of downstream target genes, and its overexpression may inhibit the proliferation of SMCs. miR-1 is involved in myocardin-dependent SMC proliferation inhibition (32). In their study, Xie *et al* suggested that miR-1 plays a critical role in the determination of SMC fate during retinoid acid-induced embryonic stem cell/SMC differentiation *in vitro* (33).

miR-21. Besides being highly expressed in ECs, miR-21 is a proproliferative and antiapoptotic regulator of VSMCs.

Recently, our group performed a series of *in vitro* and *in vivo* studies on miR-21. *In vitro* we identified that the expression of miR-21 in dedifferentiated VSMC was higher than in fresh isolated differentiated cells. Depletion of miR-21 resulted in decreased cell proliferation and increased cell apoptosis. PTEN and Bcl-2 were involved in miR-21-mediated cellular effects (34). Neointimal lesion formation is the pathological basis of AS, thus in order to investigate the correlation between miR-21 and neointimal lesions, we constructed a rat balloon injury carotid artery model. The results demonstrated

that miR-21 was aberrantly expressed in the vascular walls. Using the knock-down method it was found that modulating the overexpression of miR-21 had a significant negative effect on neointimal lesion formation (35). Following further investigation, we identified that miR-21 in VSMCs was sensitive to reactive oxygen species (ROS). miR-21 plays a role in H_2O_2-mediated gene regulation and the cell injury response through the programmed cell death 4 (PDCD4) and activator protein 1 pathways (36). Propofol is a widely used intravenous anesthetic agent with antioxidant properties. Our group investigated other mechanisms of ROS-induced injury in VSMCs, and results revealed that propofol exacerbates cell injury in VSMCs with increased ROS, partly through miRNA-21 and its target gene, PDCD4 (35).

miR-143/145. miR-143/145 is the most abundant miRNA in normal arteries and is mainly localized in VSMCs (37). It has recently been demonstrated that the downregulation of miRNA-143/145, which are coexpressed from a single promoter, regulates the switch from contractile to synthetic phenotype, allowing SMCs to migrate and proliferate (39). Serum response factor (SRF) and its coactivator, myocardin, play a key role in the control of smooth muscle phenotypes by regulating the expression of cytoskeletal genes. Using *in vivo* and *in vitro* studies, Xin *et al* confirmed that SRF and myocardin regulate a cardiovascular-specific miRNA cluster encoding miR-143/145. miR-143/145 act as integral components of the regulatory network whereby SRF controls cytoskeletal remodeling and phenotypic switching of SMCs during vascular disease (40). Furthermore, Cordes *et al* demonstrated that miR-145 is able to direct smooth muscle fate, and that miR-143 and -145 function regulates the quiescent versus proliferative phenotype of SMCs, which is able to cooperatively target a network of transcription factors, including Krüppel-like factor 4 (KLF4), myocardin and EIK-1 (41). Platelet-derived growth factor (PDGF) is one of the most potent stimuli for the migration of VSMCs. Studies have shown that PDGF mediates podosome formation in SMCs through the regulation of miR-143/-145 expression via a pathway involving Src and p53.

In a rat model of acute vascular injury, overexpression of miR-143 and -145 decreased neointimal formation, while loss of the miRNAs induced structural modification of the vessel, due to incomplete differentiation of VSMCs (42). Consistent with the above studies, we aimed to determine the exact role of miR-145, and obtained similar results to those of previous studies. Both in cultured rat VSMCs *in vitro* and in balloon-injured rat carotid arteries *in vivo*, we demonstrated that the miR-145 target KLF5 and myocardin mediate vascular neointimal growth, which may be a novel phenotypic marker and a novel phenotypic modulator of VSMCs (43).

miR-221/222. Studies by other authors and our group have confirmed the role of miR-221/222 in the regulation of VSMCs. Davis *et al* demonstrated that PDGF signaling, by modulating the expression of miR-221, regulates two critical determinants of the VSMC phenotype, including VSMC gene expression and cell proliferation. miR-221 is transcriptionally induced following PDGF treatment in primary VSMCs, leading to the downregulation of the targets, c-kit and p27 (Kip1), which directly induced cell proliferation (31,44).

Our results support the hypothesis that miR-221/-222 are novel regulators of VSMC proliferation and neointimal hyperplasia. Results of our study showed that miR-221/-222 expression was increased by growth stimulators in cultured VSMCs. Knockdown resulted in a decreased VSCM proliferation *in vitro* (45). Consistent with results by Davis *et al* (31), we also identified that as target genes, Kip1 and p57 (kip2) were involved in miR-221/222-mediated effects on VSMC growth. Using an induced rat carotid arterial model after angioplasty, we identified the overexpression of miR-221/222 in injured vascular walls, while *in vivo* knockdown suppressed VSMC proliferation (46).

Other miRNAs. Other miRNAs are also involved in mediating VSMCs. miR-29b, directly targeting DNMT3b, may mediate epigenetic regulation in AS (47). miR-146a targets KLF4 and plays a role in promoting VSMC proliferation in an *in vitro* and *in vivo* model (48). Let-7d regulates proliferation and differentiation, and RAS may also be involved (49).

4. DM-associated arteriogenesis and miRNAs

DM is recognized as a major cardiovascular risk factor. Patients suffering from diabetes and PAD are at risk of developing critical limb ischemia and foot ulceration, potentially requiring limb amputation. DM affects arteriogenesis of PAD at a number of levels. miRNAs are involved in the epigenetic regulation of key metabolic, inflammatory and antiangiogenic pathways in DM and may contribute to common disease complications. miR-126 is the most abundant miRNA in endothelial apoptotic bodies. Shedding of miR-126 from ECs has been demonstrated to regulate VEGF responsiveness and to confer vascular protection. miR-126 may contribute to VEGF resistance and endothelial dysfunction in DM (50). miR-208 is involved in insulin-induced VSMC proliferation via the downregulation of its potential target, p21, a key member of the cyclin-dependent kinase inhibitory protein family (51,52). miR-221 regulates a high glucose-induced endothelial dysfunction. Under hyperglycemic conditions, miR-221 triggers the inhibition of c-kit and impairment of HUVECs migration (53). In high glucose- and AGE-mediated vascular damage, miR-221/-222 targeting Kip1 and Kip2 regulate vascular cell proliferation (54). Wang *et al* revealed that miR-320 is associated with impaired angiogenesis in DM. Transfection of miR-320 inhibitor may be a therapeutic approach for the treatment of impaired DM-associated angiogenesis (55).

5. miRNAs and PAD

Ischemic complications are the leading cause of morbidity and mortality in PAD patients. An improved understanding of the molecular mechanisms of limb ischemia is required to improve therapeutic options. miR-92a has been identified as an endogenous repressor of the angiogenic program. Overexpression of miR-92a blocks angiogenesis and vessel formation. Based on this, using a mouse hind-limb ischemia model, Bonauer *et al* identified that ischemic injury significantly increased the expression of miR-92a, and injection of antagomir-92a reduced necrosis, improved perfusion and caused functional recovery of ischemic limbs. These results suggest that in the setting of

ischemic disease, miRNA-92a, targeted by several proangiogenic proteins including subunit α5, controls angiogenesis and the functional recovery of ischemic tissues (56). Grundmann *et al* found that miRNA-100 was significantly downregulated following the induction of hind-limb ischemia in mice. It was demonstrated that miR-100 has an antiangiogenic function and represses the mammalian target of rapamycin (mTOR) signaling *in vitro*, which modulates proliferation and tube formation (57). miR-322/-424 is a critical mediator of oxygen-dependent changes in ECs and is physiologically upregulated in tissues undergoing vascular remodeling and angiogenesis. Hypoxia differentially increased miRNA-424 levels in ECs and miR-424 targeted cullin 2 (CUL2), which plays an important physiological role in post-ischemic vascular remodeling and angiogenesis (27).

In addition to the above basic investigations concerning PAD, there is now a focus on clinical studies. Arteriosclerosis obliterans (ASO) is a type of PAD. Wang *et al* (58) investigated the role of miR-21 in human arteries and VSMCs with ASO. The results of those authors suggested that miR-21 is capable of regulating ASMC function by targeting tropomyosin 1, and the hypoxia inducible factor-1α/miR-21/tropomyosin 1 pathway may play a critical role in the pathogenesis of ASO (58). Specific signatures of miRNAs may be obtained from total serum/plasma or tissues and are able to be used in the identification of biomarkers for the diagnosis, prognosis or even the etiology of a disease (59). Currently, miRNAs have become a hot topic as biomarkers and therapeutic targets for cardiovascular disease (60). Caporali *et al* (59) revealed that miR-503 was highly expressed in ischemic muscle specimens and the plasma of diabetic patients with critical limb ischemia. Their study provided evidence for a role of miR-503 in DM-induced endothelial defects contributing to impaired postischemic angiogenesis, and also demonstrated that the overexpression of miR-503 inhibited EC proliferation, migration and network formation, and reduced VSMC proliferation and migration. Thus, miR-503 may be considered a suppressor of postischemic neovascularization in DM and a potential therapeutic target for improving healing of diabetic ischemic tissues (61). In order to identify a specific and sensitive biomarker for ASO, using tissue samples and blood samples obtained from ASO patients, Li *et al* examined the expression levels of a series of miRNAs. The results identified a significant increase in miR-130a, miR-27b and miR-210 expression, which was positively correlated with the Fontaine stage (59). Therefore, the serum and tissue levels of these miRNAs serve as potential biomarkers for early stage PAD.

6. Conclusion

At present, investigating cellular and molecular mechanisms and gene therapy have become hot topics in PAD. Accumulating evidence has demonstrated that multiple miRNAs may serve as novel biomarkers and new therapeutic targets through their important roles in regulating cell proliferation, differentiation and apoptosis. However, these roles should be further investigated in miRNA-based therapy. As a single miRNA is able to regulate a number of target genes, while multiple miRNAs may be regulated by single genes, more studies are required to analyze the complex interactions between specific miRNAs and their targets during PAD. In addition, the detailed cellular and molecular mechanisms of these specific miRNAs should be further investigated to identify ways of preventing and treating PAD.

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