Angiopoietin-Like Proteins

— Potential Therapeutic Targets for Metabolic Syndrome and Cardiovascular Disease —

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Recent major increases in obesity and related metabolic diseases (known as the metabolic syndrome (MetS)) because of sedentary lifestyles and overnutrition in developed and developing countries, are an exploding medical and social problem. These conditions are associated with increased risk of cardiovascular disease (CVD), the leading cause of death. Thus, it is necessary to understand the molecular basis underlying MetS and develop effective preventive and therapeutic approaches against CVD. To date, 7 angiopoietin-like proteins (Angptls) that are structurally similar to angiopoietins have been identified. However, none binds to the angiopoietin receptor, Tie2, or to the closely related Tie1 receptor, suggesting that these ligands function differently from angiopoietins. Some Angptls potently regulate angiogenesis, similar to angiopoietins, whereas others have pleiotropic activity other than angiogenesis and function in lipid and energy metabolism. In this review, we focus on the roles of Angptl2 and Angptl6/angiopoietin-like growth factor (AGF) in the development of MetS and CVD, and discuss the potential for Angptl2 and Angptl6/AGF to function as molecular targets for the prevention and treatment of both conditions. (Circ J 2009; 73: 2192–2197)

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besity and related metabolic diseases (known as the metabolic syndrome (MetS)) are associated with increased risk of cardiovascular disease (CVD). Thus, it is necessary to both understand the underlying molecular basis and develop effective preventive and therapeutic approaches. We and others have independently identified 7 angiopoietin-like proteins (Angptls), namely, Angptl1/angiopoietin-related protein 1 (ARP1)/angioarrestin,1,2 Angptl2/ARP2,3 Angptl3,4,5 Angptl4/ARP4/PPARγ angiopoietin-related (PGAR)/fasting-induced adipose factor (FIAF)/hepatic fibrinogen/angiopoietin-related protein (HFARP) 6,9 Angptl5,10,11 Angptl6/angiopoietin-like growth factor (AGF),12 and Angptl7.13 These Angptls are structurally similar to angiopoietins, which are characterized by a coiled-coil domain at the N-terminus and a fibrinogen-like domain at the C-terminus. In addition, the N-terminus Angptls exhibit a highly hydrophobic region reminiscent of a signal sequence for protein secretion. Cells transfected with Angptl expression vectors secrete Angptl protein into culture supernatants,1,8,14 and Angptls 2,3,4, and 6 have all been detected in the systemic circulation, suggesting that some Angptls function in an endocrine manner in vivo.1,9,15–19 Whereas angiopoietins act via the Tie2 receptor tyrosine kinase, the signaling of which regulates vascular stabilization and remodeling, Angptls do not bind to either Tie2 or the related Tie1 receptor, suggesting that these orphan ligands function differently from angiopoietins. Although several studies show that Angptls potently regul-
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Angptl6/AGF and the MetS

Abundant Angptl6/AGF expression is restricted to the liver in humans. In mice, although it is also expressed abundantly in the liver, Angptl6/AGF expression is detected in the brain, heart, skeletal muscle, kidney, and some hematopoietic cell lineages. Angptl6/AGF protein is also detected in the serum of both mice and humans, and functions in angiogenesis and to increase systemic energy expenditure in an endocrine manner. In mice, reverse transcriptase/polymerase chain reaction analysis of full-length Angptl6/AGF indicates its expression is restricted to the liver, whereas amplification with primers targeting exons 4–6 reveals a broader expression. These results suggest that, in mice, the liver is the major source of full-length Angptl6/AGF in serum, because the signal peptide is present only in the full-length Angptl6/AGF, not in the shorter protein. Further studies are needed to clarify whether the short-form Angptl6/AGF exists and, if so, how it functions.

Mice deficient in full-length Angptl6/AGF (ie, Angptl6/AGF knock-out (KO) mice) show marked obesity because of decreased energy expenditure and insulin resistance. Conversely, transgenic mice in which full-length Angptl6/AGF expression is constitutively and broadly driven by the CAG promoter (chicken β-actin promoter with the cytomegalovirus immediate early enhancer; CAG-Angptl6/AGF mice) exhibit a lean phenotype with increased energy expenditure and are protected against diet-induced obesity and insulin resistance. K14-Angptl6/AGF mice overexpressing Angptl6/AGF in the skin, which exhibit increased serum levels of Angptl6/AGF, as high as those seen in CAG-Angptl6/AGF transgenic mice, show less body fat than wild-type controls. Moreover, adenoviral overexpression of Angptl6/AGF in the liver results in increased serum levels of Angptl6/AGF and amelioration of diet-induced obesity and insulin resistance. Taken together, full-length Angptl6/AGF in the circulation seems to increase systemic energy expenditure.

To date, however, the evidence indicates that circulating levels of Angptl6/AGF are likely to be paradoxically elevated in obese or diabetic conditions; serum levels are elevated in obese mice and humans (unpublished data), as well as in patients with diabetes. Furthermore, the levels of Angptl6/AGF have been independently and positively correlated with fasting levels of serum glucose. These findings raise the following questions: (1) why do elevated energy expenditure, (2) is Angptl6/AGF upregulated to compensate for excess body fat, and (3) does Angptl6/AGF resistance occur because of downregulation of its receptor or downstream signaling. To answer these questions needs further studies elucidating the mechanism that regulates Angptl6/AGF expression in the liver and circulation, and also identifying its cognate receptor.

The most likely target tissue for Angptl6/AGF is skeletal muscle, because gene expression related to energy expenditure, such as that of PGC-1α and PPARδ, is downregulated in Angptl6/AGF KO mice and upregulated in CAG-Angptl6/AGF mice. Furthermore, Angptl6/AGF binds to C2C12 myocytes and stimulates phosphorylation of p38 MAPK, which directly enhances the stability and activation of PGC-1 protein. On the other hand, food intake by Angptl6/AGF KO mice is slightly higher than that seen in wild-type controls, although not statistically significant, suggesting Angptl6/AGF may act on the hypothalamus to increase peripheral energy expenditure and suppress appetite, similarly to leptin. Indeed, acute administration of recombinant Angptl6/AGF protein into the intracranial ventricle significantly decreased food intake in mice (unpublished data). Angptl6/AGF was also reported to suppress gluconeogenesis, as well as expression of glucose-6-phosphatase, through an PI3K/Akt/FoxO1-dependent pathway in rat hepatocytes. These findings are compatible with our in vivo findings that the fasting level of glucose is increased in Angptl6/AGF KO mice but decreased in diet-induced obese mice administered adenovirus expressing Angptl6/AGF. Because Angptl6/AGF is mainly expressed by hepatocytes, it may act on hepatocytes to suppress gluconeogenesis in an autocrine/paracrine manner. This possibility partially accounts for the insulin-sensitizing effect of Angptl6/AGF. Taken together, the studies establish Angptl6/AGF as a potential target for development of pharmacologic interventions to counteract obesity and insulin resistance. Further studies, especially to identify the Angptl6/AGF receptor, are needed to clarify Angptl6/AGF function.

Recently, the association between Angptl6/AGF gene polymorphisms and MetS-related phenotypes was examined in subjects from the population-based French MONICA Study (n=3,402). One single-nucleotide polymorphism (SNP), the G allele of rs6511435, tended to be associated with lower plasma levels of glucose (P=0.009). Also, obese subjects carrying another SNP, a G allele of rs6511435, had higher plasma levels of insulin than did AA subjects (P=0.0055). Moreover, the G allele of rs6511435 tended to be associated with a 20% higher risk of MetS (P=0.034). When false discovery rate testing (40 tests) was applied, these associations were no longer statistically significant; nonetheless, a weak association of polymorphisms with these parameters cannot be excluded. Further studies are needed to arrive at definite conclusions.

Angptl6/AGF and Ischemic Vascular Disease

Angptl6/AGF was first identified as a proangiogenic factor when it was shown that microvessel density was increased in the skin of K14-Angptl6/AGF mice (Figure 1). Recombinant Angptl6/AGF protein had a chemoattractive effect on endothelial cells in Matrigel plug and corneal neovascularization assays, 2 independent indicators of de novo angiogenesis. Conversely, blood flow was reduced in the hindlimbs of Angptl6/AGF KO mice compared with wild-type controls. Recently, we found that Angptl6/AGF enhances blood flow in a mouse hindlimb ischemia model and we defined the mechanisms underlying Angptl6/AGF signaling in endothelial cells. Intramuscular injection of adenovirus expressing Angptl6/AGF into the ischemic limb induced Angptl6/AGF production, which ameliorated blood flow through induction of angiogenesis and arteriogenesis, reducing the necessity for limb amputation. Our in vitro analysis showed that exposing human umbilical venous
endothelial cells to Angptl6/AGF increased nitric oxide (NO) production through activation of ERK1/2-endothelial NO synthetase (eNOS) signaling. Angptl6/AGF-induced eNOS phosphorylation, NO production, and endothelial cell migration were all abolished by specific MEK1/2 inhibitors. Moreover, Angptl6/AGF did not restore blood flow to ischemic hindlimbs of mice treated with the NO synthetase inhibitor, NG-nitro-L-arginine methyl ester hydrochloride, or in eNOS knockout mice, indicating that Angptl6/AGF-mediated NO production is required to increase blood flow. Activation of an ERK1/2-eNOS-NO pathway is a crucial signaling mechanism by which Angptl6/AGF increases blood flow through induction of angiogenesis and arteriogenesis. These findings suggest that enhancement of Angptl6/AGF signaling might be a good strategy for ameliorating ischemic vascular disease.

Angptl2 and MetS

Angptl2 was first reported as a secreted protein with a weak stimulatory effect on endothelial cell sprouting in vitro. We previously reported that Angptl2 functions in the vascular development in zebrafish and also that Angptl2 has an anti-apoptotic effect on human venous endothelial cells. We recently reported that adipocyte-derived Angptl2 is a key mediator linking obesity to adipose tissue inflammation and systemic insulin resistance. Angptl2 is abundantly expressed in adipose tissues, especially visceral adipose tissues. Both the expression and serum concentration of Angptl2 are increased in obese mice, most likely because of hypoxia and endoplasmic reticulum (ER) stress occurring in the adipose tissue. In humans, the level of Angptl2 in the circulation appears to be upregulated in obesity and related metabolic diseases. Serum levels of Angptl2 are positively correlated with body mass index, abdominal circumference, and the level of serum C-reactive protein (CRP) in healthy volunteers. Serum levels of Angptl2 in diabetic patients are higher than those in healthy controls. In diabetic patients, serum levels of Angptl2 positively correlate with the visceral fat area estimated by MRI, the homeostasis model assessment of insulin resistance index, and serum levels of CRP, and negatively correlate with the M value calculated by the hyperinsulinemic euglycemic glucose clamp method. Furthermore, the plasma level of Angptl2 decreased in parallel with the reduction of visceral fat in obese diabetic patients treated with pioglitazone, a PPARγ agonist with unique anti-diabetic activity that decreases visceral fat, suppresses inflammation, and ameliorates insulin sensitivity. These findings indicate that visceral fat may be the primary source of Angptl2 in the circulation in humans. In addition, Angptl2 mRNA expression in cultured 3T3-L1 adipocytes was halved 24h after the addition of a PPARγ agonist to the medium (unpublished data), which may explain in part the reduction of plasma levels of Angptl2 by pioglitazone treatment. These results are compatible with the fact that suppressing Angptl2 ameliorates insulin sensitivity in mice. The anti-diabetic effect of pioglitazone may be partially related to a
reduction of Angptl2 production.

We have found that overexpression of Angptl2 in skin tissue results in local inflammation represented by a red and swollen appearance, abundant leukocytes attached to the wall of post-capillary venules (Figure 1), and increased blood vessel permeability. Interestingly, the number of blood vessels remains unaltered, suggesting that Angptl2 promotes vascular inflammation but not angiogenesis in vivo. Because Angptl2 promotes angiogenesis in avascular tissue, such as the cornea, it seems it acts on vascular endothelial cells differently from other tissues. In adipose tissue, Angptl2 appears to induce vascular inflammation, but not angiogenesis, in the same way as it does in skin tissue. Adipose-tissue-specific Angptl2 transgenic mice show vascular inflammation, increased macrophage infiltration and increased inflammatory cytokine expression, even though they are not obese. Conversely, Angptl2 KO mice fed a high-fat diet show fewer infiltrated macrophages and decreased expression of inflammatory markers compared with wild-type mice. These phenotypes are explained by the ability of Angptl2 to induce an inflammatory cascade in vascular endothelial cells through α5β1 integrin receptors, as well as to promote chemotactic activity in monocytes through α4 and β2 integrin receptors. Recently, it was reported that inputs from leukocyte integrin signaling modulate the Toll-like receptor 4 (TLR4) signaling, which functions in innate immune responses. Recent studies implicate the TLR4 pathway in the mechanism of chronic inflammatory diseases, such as inflammation in obese adipose tissue and atherosclerosis. Furthermore, Angptl2 has a fibrinogen-like domain at the C-terminus, and fibrinogen is reported to act as an intrinsic TLR4 ligand on monocytes, suggesting that Angptl2 may also function as a TLR4 ligand. Further studies are needed to clarify whether there is a specific Angptl2 receptor expressed by endothelial cells and/or monocytes.

Angptl2 and CVD

Both the serum and plasma levels of Angptl2 in patients with coronary artery disease (CAD) are higher than those of healthy subjects. Furthermore, plasma levels of Angptl2 are even higher in patients with multivessel CAD than in those with single-vessel disease, based on our coronary angiography results (Figure 2), although further study with larger numbers of patients is needed to confirm this result. Because Angptl2 promotes vascular inflammation, most likely via the integrin α5β1/Rac1/NF-κB pathway, and because vascular injury accompanied by inflammation is considered an early feature of arteriosclerosis, circulating Angptl2 may be directly involved in the initiation and progression of atherosclerosis. In addition, endothelial cells from segments of the internal mammary arteries from smokers with CAD express higher levels of Angptl2 mRNA than tissues from nonsmokers with the same disease. Because smoking is closely associated with the development of inflammation and increased risk of atherosclerosis, Angptl2 may be the mediator linking smoking and CVD.
Moreover, serum levels of Angptl2 positively correlate with the serum level of CRP, which is a highly effective marker of atherosclerosis risk, but apparently do not cause atherosclerosis.\textsuperscript{44} Circulating Angptl2 might likewise be used as a good risk marker for CVD and more importantly, it is of great interest to determine whether Angptl2 causes atherosclerosis, so further studies are needed to determine both these possibilities. A randomized double-blind mega-study, PROActive, showed that pioglitazone treatment decreased the development of cardiovascular events in high-risk, type 2 diabetic patients.\textsuperscript{36,45} The reason for this reduction remains unclear, but amelioration in control of blood glucose and systemic insulin sensitivity likely contributed to this outcome. Because plasma levels of Angptl2 decrease with pioglitazone treatment, reduced Angptl2 production may account for that finding. Blocking Angptl2 signaling may be beneficial in preventing and treating CVD.

**Prospective Roles of Angptl2 in Other Lifestyle Diseases**

Inactivity and obesity trigger persistent, low-grade systemic inflammation, which is linked to the development of chronic lifestyle diseases in some tissues. We recently reported that adipocyte-derived Angptl2 is a key mediator linking obesity to adipose tissue inflammation and systemic insulin resistance.\textsuperscript{22} Expression of Angptl2 is not restricted to adipose tissues, suggesting its role in other diseases, particularly those associated with chronic inflammatory conditions, such as diabetic microvascular complications and cancer.

Upregulation of Angptl2 mRNA and protein has been observed in the glomeruli of patients with diabetic nephropathy, and this observation closely correlates with abnormal microvascularature and endothelial inflammation.\textsuperscript{46} Because both vascular endothelial injury and leukocyte recruitment including monocytes are considered crucial steps in diabetic nephropathy,\textsuperscript{47,48} Angptl2 may play an important role in the pathogenesis of this condition. Similarly, Angptl2 concentration in the aqueous humor is significantly increased in patients with diabetic retinopathy (unpublished data), which is predictable because we have found that Angptl2 expression is upregulated by hypoxia. Because Angptl2 induces vascular inflammation and increases vascular permeability, both of which potentially exacerbate the pathological state of diabetic retinopathy, it is of great interest to determine whether Angptl2 functions in the development and progression of diabetic microvascular complications, including diabetic retinopathy and diabetic nephropathy.

Hypoxic conditions occur under physiological circumstances and are also common in pathologic states, including not only diabetic retinopathy,\textsuperscript{49} but also tumor growth.\textsuperscript{50,51} Recently, it was reported that carcinoma-associated fibroblasts (CAFs), which play an important role in tumor growth and metastasis,\textsuperscript{52} express Angptl2 in some tumors that are refractory to anti-VEGF therapy.\textsuperscript{53} We have also observed that Angptl2 is expressed by some cancer cells as well as CAFs (unpublished data). Both hypoxia and ER stress increase the expression of Angptl2, and both conditions are commonly observed in chronic inflammatory states, including inflammation in obese adipose tissue and in cancer tissue. Thus, Angptl2 signaling may be a common key player in these 2 major diseases related to chronic low-grade inflammation. Obesity is known to increase the risk of several cancers, such as esophageal,\textsuperscript{54,55} colon,\textsuperscript{54,55} liver,\textsuperscript{56} gall bladder,\textsuperscript{55} pancreatic,\textsuperscript{55,57} kidney,\textsuperscript{55} prostate,\textsuperscript{58} breast,\textsuperscript{54,55} and uterine endometrial cancers.\textsuperscript{54,55} Circulating inflammatory mediators, upregulated in obesity, including Angptl2, could be involved in carcinogenesis.

**Conclusions**

In this review, we have focused on the roles of Angptl6/AGF and Angptl2 in MetS and CVD. Although both Angptl3 and Angptl4 in the Angptl family are known to regulate lipid metabolism in function in CVD,\textsuperscript{59–61} we have not described their activities in detail here. Nonetheless, it is of interest to determine whether other Angptl family members contribute to CVDs and MetS. As shown in Figure 1, significant differences are observed in the vascularization of K14-Angptl2 and K14-Angptl6/AGF mice, suggesting the existence of specific cognate receptors. Finally, we propose that enhancement of Angptl6/AGF signalng or suppression of Angptl2 signaling could be an effective strategy against obesity and obesity-related diseases (Figure 3). To develop useful drugs of this type, further studies are necessary to elucidate how these factors are expressed and to identify their cognate receptors.

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