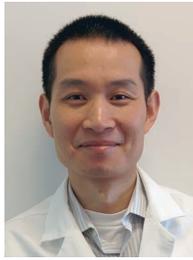


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AIBP: A NOVEL MOLECULE AT THE INTERFACE OF CHOLESTEROL TRANSPORT, ANGIOGENESIS, AND ATHEROSCLEROSIS

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Abstract

Cardiovascular disease, which is often driven by hypercholesterolemia and subsequent coronary atherosclerosis, is the number-one cause of morbidity and mortality in the United States. Based on long-term epidemiological studies, high-density lipoprotein cholesterol (HDL-C) levels are inversely correlated with risk for coronary artery disease (CAD). HDL-mediated reverse cholesterol transport (RCT) is responsible for cholesterol removal from the peripheral tissues and return to the liver for final elimination.¹ In atherosclerosis, intraplaque angiogenesis promotes plaque growth and increases plaque vulnerability. Conceivably, the acceleration of RCT and disruption of intraplaque angiogenesis would inhibit atherosclerosis and reduce CAD. We have identified a protein called apoA-I binding protein (AIBP) that augments HDL functionality by accelerating cholesterol efflux. Furthermore, AIBP inhibits vascular endothelial growth factor receptor 2 activation in endothelial cells and limits angiogenesis.² The following discusses the prospect of using AIBP as a novel therapeutic approach for the treatment of CAD.

Vasa Vasorum and Plaque Growth

Vasa vasorum are the microvessels that underlie the adventitial layer of large arteries or veins and provide oxygen and nutrients to these major vessels. Emerging evidence suggests that atherosclerotic plaque growth is closely associated with intraplaque angiogenesis, which occurs from vasa vasorum. Local administration of fibroblast growth factors (FGF), a potent angiogenic factor that promotes angiogenesis in the adventitia, enhanced the growth of atherosclerotic lesions in apoE-deficient mice.³ In rat models receiving injury to the arterial walls, administration of vascular endothelial growth factor (VEGF) exacerbated neointimal thickening, although VEGF alone was not sufficient to initiate the thickening.⁴ One important component that instigates intraplaque angiogenesis is inflammation, which causes oxidative stress. For example, transgenic mice—which ectopically express p22-phox, a major subunit of NAD(P)H oxidase in the smooth muscle cells—will generate increased oxidative stress in carotid lesions, further contributing to angiogenesis and plaque progression.⁵ It is posited that plaques behave like the tumor microenvironment in which inflammatory cells and/or other cells secrete a plethora of angiogenic factors to induce angiogenesis. Due to hypoxia, the intraplaque microenvironment often results in vascular leakage, which further contributes to intraplaque hemorrhage, elevated lipid accumulation, and exacerbated inflammation. All of these effects result in the formation of vulnerable plaques. Interestingly, the perivascular microvessels also serve as niches for multipotent pericytes and endothelial progenitor cells. These cells play a critical role in augmenting intraplaque angiogenesis by providing endothelial cells (ECs) or supporting cells for angiogenic growth.⁶

Reverse Cholesterol Transport, Lymphatics, and CAD

Mounting evidence suggests that lymphatics play an essential role in lipid transport. Chylomicrons, a form of dietary lipids, are transported to the blood via intestinal lymphatic vessels.⁷

Similarly, the transport of high-density lipoproteins (HDL) from the peripheral tissues back to plasma depends on lymphatic vasculature but not on venous capillaries. It has been shown that there is roughly 344 mg of HDL transported in human peripheral lymph daily.⁸ Inactivation of one copy of VEGFR3 in mice diminishes lymphatics in the skin but retains a normal blood vasculature. Accordingly, these mice demonstrate attenuated ability to remove subcutaneous fat.⁹ Lymphatic vessels are found in the adventitia of arteries and are in close proximity to the blood vessels.¹⁰ Surgical disruption of lymphatic vessels, by using aortic transplantation as well as blocking lymphatic regrowth using VEGFR3-neutralizing antibody, led to a remarkable increase in aortic lipid accumulation.¹¹

Lymphatic malfunction contributes to the pathogenesis of atherosclerosis, cardiovascular disease, and diabetes. The connection between atherosclerosis and impaired lymphatic functions has been observed for a long time.^{12,13} Experimental evidence has recently emerged suggesting that lymphatic vasculature provides a protective pathway for lipid and inflammatory cell removal from the arterial wall and peripheral tissues and thus alleviates the development of lipid deposition.^{11,14} It remains unclear whether lymphatic vessels play an active role, which is speculated to occur via SR-BI-mediated transendocytosis, or a passive role, where it functions merely as a collecting conduit.

HDL in the Regulation of Angiogenesis and Lymphangiogenesis

Numerous reports indicate that HDL associated bioactive lipids and HDL-evoked changes in cholesterol levels regulate angiogenesis and lymphangiogenesis. In ECs, cholesterol is mostly enriched in lipid rafts of the cell membrane, where it is essential for cell signaling and maintenance of regular cellular functions such as migration and proliferation.¹⁵ Manipulation of cholesterol levels in ECs to decrease lipid rafts would interfere with membrane receptor-like VEGF receptor 2 (VEGFR-2) signaling

and subsequently impair angiogenesis. Notably, other molecules involved in angiogenesis such as FGFR, ICAM-1, and VCAM-1 also localize to membrane lipid rafts and may be affected.^{16,17}

Sphingosine-1-phosphate (S1P), which docks on HDL via apoM, exerts an effect on both angiogenesis and lymphangiogenesis.¹⁸ The effects of HDL-associated S1P on angiogenesis seem to be context-dependent. For example, S1P on HDL stimulates *in vitro* angiogenesis as well as ovarian angiogenesis in the follicular fluid via ERK, PKC, Akt, and eNOS activation.¹⁹ Endothelial lipase is shown to play a critical role in S1P-induced aortic ring neovascularization, presumably by releasing free S1P from HDL.²⁰ In contrast, another study tested the effect of different concentrations of HDL on ECs and found a biphasic effect of HDL on endothelial progenitor cell (EPC)-mediated *in vitro* angiogenesis. High levels of HDL inhibit angiogenesis through stimulation of Rho-associated kinase (ROCK) and inhibition of the Akt and ERK pathways.²¹ *In vivo*, S1P receptor deficiency increases while S1P receptor activation decreases angiogenesis. Further, S1P signaling in ECs preserves vascular integrity by stabilizing adherent junctions between neighboring ECs and responds to laminar shear stress to transduce blood flow-regulated EC homeostasis.^{22,23}

S1P promotes human lymphatic endothelial cell (LEC) tube formation and cell migration *in vitro* and facilitates growth of lymphatic vessels in the Matrigel plug *in vivo*.²⁴ Mechanistically, it promotes lymphangiogenesis through stimulation of PLC γ and calcium signaling. S1P levels are increased in breast cancer patients, in whom lymphatic vasculature is essential for tumor dissemination. Similarly, sphingosine kinase, a kinase that produces S1P, has increased expression levels and supports lymphangiogenesis in the tumor microenvironment.²⁵

Despite many papers ascribing the actions of S1P to its receptor, HDL, at least in part, is involved in S1P-induced signaling. For example, SR-BI, the HDL receptor, is shown to regulate S1P-induced upregulation of NF- κ B and cell adhesion molecules such as VCAM-1 and ICAM-1 via activation of PI3K and eNOS.²⁶ Of note, the receptors for S1P, G protein-coupled receptors, localize and activate in lipid rafts.²⁷ Whether targeted cholesterol efflux mediated by AIBP regulates the signals initiated from S1P is an intriguing question that merits further study.

Zebrafish: a Unique Animal Model for the Study of Atherosclerosis, Angiogenesis, and Lymphangiogenesis

Through our series of studies, we have established that zebrafish fed a high cholesterol diet (HCD) are a novel animal model to study the early atherosclerogenesis, when foam cell formation occurs. The advantages of using zebrafish include its optical transparency, easy genetic manipulation, generation of a large quantity of progeny per breeding, short life cycle, and rapid development of diseases. When fed an HCD similar to that used for mouse atherosclerosis studies (4% cholesterol weight for weight in food) for 2 months, adult zebrafish develop early healed lesions in the dorsal aorta.²⁸ These lesions revealed lipid accumulation, macrophage retention, EC inflammation, and smooth muscle cell proliferation. All of these features are found in early human atherosclerotic lesions. Oxidation of low-density lipoprotein (LDL) renders it proinflammatory, and oxidized LDL (OxLDL) is believed to be one of the key culprits that promote atherosclerosis. There is a 20-fold increase in OxLDL levels in zebrafish fed an HCD compared to control diet, and the absolute levels are much higher than that previously observed in humans.²⁸ The OxLDL levels are measured using an ELISA assay based on E06, a monoclonal antibody against oxidized phospholipids

developed by the Witztum lab.²⁹ Consistent with this data, liquid chromatography tandem mass spectrometry (LC-MS/MS) measurement of the levels of oxidized cholesterol esters and phospholipids indicates a 10- to 70-fold increase, further supporting the use of zebrafish for studying atherosclerosis and lipid oxidation.³⁰ The epitope recognized by E06 is also found on zebrafish HDL; this epitope is rarely found in human HDL. Yet there are reports on human apoA-I oxidation,³¹ so it is possible that the modified apoA-I is trapped in the intima and precludes its detection in human plasma.³²

After the proof-of-concept studies, the zebrafish larvae were used for further analyses because of the aforementioned advantages. Feeding transgenic zebrafish larvae an HCD for 2 weeks also resulted in oxidation-specific epitopes (OSE) accumulation and vascular inflammation, which increase vascular leakage and macrophage recruitment.³³ We created a transgenic zebrafish with conditional expression of EGFP-tagged single chain monoclonal antibody IK17, which targets malondialdehyde-modified epitopes, to monitor the spatiotemporal accumulation of OSE. Furthermore, the expression of IK17-EGFP reduced vascular lipid accumulation.³³ Thus, from a pathogenesis point of view, the initial events of atherosclerosis are conserved between zebrafish and humans.

Zebrafish angiogenesis proceeds in a similar manner to humans, and the genetic pathways governing angiogenesis such as VEGF and Notch are conserved in zebrafish. Several transgenic zebrafish lines such as EC-specific promoter driving fluorescent protein expression—including Tg(fli1:EGFP) and Tg(flk1:mcherry-RAS)—have been extensively used for zebrafish angiogenesis studies.³⁴ These transgenic zebrafish permit a spatiotemporal and real-time examination of the development of *in vivo* angiogenesis for which fine-tuned mechanisms are delineated. Using the two transgenic zebrafish lines, we deciphered the role of AIBP in regulation of angiogenesis as discussed above in detail.

Zebrafish have also been employed to study lymphangiogenesis, although it appears that factors such as Prox1, Coup-TFII, and Sox18 that are essential for mouse lymphatic vessel growth seems to be dispensable for zebrafish lymphangiogenesis.³⁵ Nonetheless, people have used different lymphatic reporter fish lines to study the functionality of the lymphatic system in zebrafish, and the key features are congruent with their mammal counterparts. For example, lymphatics in zebrafish are important for removal of subcutaneous fluids, and defects in lymphatics result in edema.^{36,37} The differential mechanism controlling zebrafish lymphatic vessel development may be adopted to treat lymphedema in humans.

AIBP-Regulated Cholesterol Efflux, Angiogenesis, and Atherosclerosis

Cholesterol is essential for normal cell function, from maintenance of structural integrity to biogenesis of hormones and ligands. However, excessive cholesterol is detrimental to cells. Cholesterol efflux enables the clearance of excess cholesterol from cells and is essential for maintenance of cholesterol homeostasis.³⁸⁻⁴⁰ Cholesterol efflux is carried out by cholesterol transporters such as ATP-binding cassette family members A1 (ABCA1) and G1 (ABCG1), and scavenger receptor class B member 1 (SR-BI) in an ATP-dependent manner.⁴¹⁻⁴⁵ ABCA1 resides on the cell membrane and transports cholesterol to lipid-poor apoA-I or nascent HDL, while ABCG1 is located in the cytosol and deploys cholesterol to HDL. Lack of ABCG1 facilitates *ex vivo* angiogenesis of a murine aortic ring. SR-BI is a receptor for HDL and mediates

selective uptake of cholesterol esters on HDL.⁴⁶ Interestingly, SR-BI favors cholesterol efflux from a phospholipid-rich environment.⁴⁷ In addition, SR-BI is located in lipid rafts and was recently indicated as a sensor to regulate cholesterol homeostasis in the membrane.⁴⁸ It is also reported to engage in active lymphatic transport of interstitial HDL.¹⁴

High levels of HDL may be beneficial because it carries excess cholesterol away from cells. HDL-C in epidemiological studies is inversely associated with CVD risk. Infusion of wild type apoA-I, apoA-I Milano, or its mimetic peptides into rabbits or mice mitigates atherosclerosis.^{49,50} Thus, different approaches have been applied to increase HDL-C levels such as CETP inhibitors and RVX-208, a compound that functions as an inhibitor for BET bromodomains to increase apoA-I protein synthesis.^{51,52} However, clinical trials at the Cleveland Clinic with RVX-208 failed to document an increase in apoA-I protein levels in humans compared to placebo control. So far, the majority of clinical trials aiming to reduce risks for CAD by increasing HDL-C levels have failed to show efficacy.^{53,54} Moreover, the genetic evidence supporting the cause-and-effect relationship between higher HDL-C levels and lower CVD risk is controversial.⁵⁵ To address the discrepancy, several investigators proposed that augmenting the ability of cholesterol removal by HDL instead of increasing HDL-C levels would improve the outcome of CVD.⁵⁶ Indeed, large-scale measurement of the ability of LDL-depleted plasma to facilitate cholesterol efflux confirmed lower risk for prevalent atherosclerotic burden and CAD in patients with higher cholesterol efflux capacity.⁵⁷⁻⁵⁹

We have recently reported that AIBP demonstrates a capability to enhance cholesterol efflux of HDL and therefore may fulfill the role of boosting HDL functionality (Figure 1).² AIBP is a secreted protein discovered in a screen of proteins that interact with apoA-I.⁶⁰ Human AIBP is abundantly expressed in a majority of tissues that govern secretions. Of particular interest, it is expressed in the vascular smooth muscle and heart muscle but not in skeletal muscle.⁶¹ The protein localization may imply its role in cardiovascular functions. In addition, AIBP was detected in human cerebrospinal fluid and urine but not in the plasma of healthy subjects.⁶⁰ The human apoAIBP gene resides on the chromosome region associated with familial combined hyperlipidemia, which may cause premature CAD.⁶² Before our studies, there were no publications documenting the role of AIBP in lipid metabolism and cardiovascular disease.⁶³ We found that an incubation of AIBP with HDL increased cholesterol efflux to HDL in ECs. The ability of AIBP to promote cholesterol efflux plateaus after 6 hours. One reason for this plateau may be the relatively small amount of AIBP used (the molar ratio of AIBP to HDL is 75). Since HDL is documented to trigger AIBP release from cells,⁶⁰ it is possible that AIBP released from HDL-supplemented cells will catch up with the effect caused by trace amounts of AIBP. Another reason for the plateau may be due to an insufficient HDL cholesterol deposition pool for AIBP to carry out its function in an in vitro system. Under in vivo conditions, we do not expect such a problem because a much larger pool of HDL is present. We showed that this change of cholesterol efflux is a spatiotemporal-dependent event that occurs in development during zebrafish angiogenesis.²

Most studies on cholesterol efflux associated with atherosclerosis were done in macrophages, so whether AIBP accelerates cholesterol efflux from these particular cells merits further investigation. Additionally, maintenance of cholesterol homeostasis in ECs appears critical for proper vascular function. Increased cholesterol levels in ECs can result in elevated levels of lipid rafts and their

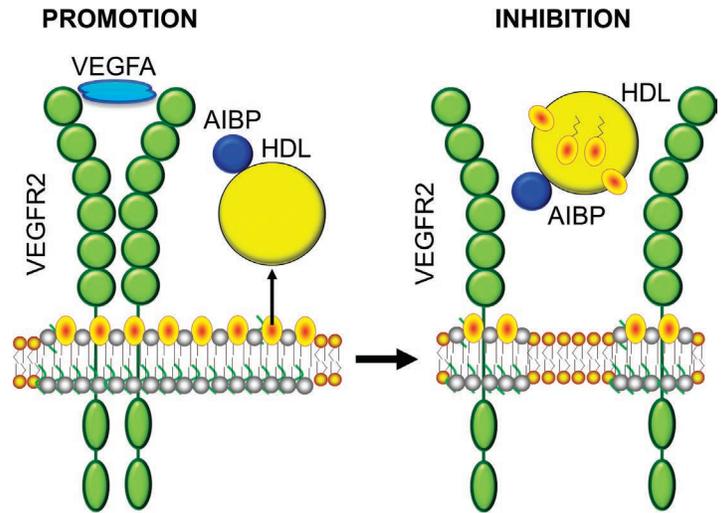


Figure 1. AIBP-mediated targeted cholesterol efflux from endothelial cells disrupts caveolae/lipid rafts and inhibits VEGFR2 signaling. VEGFA-stimulated VEGFR2 signaling induces the receptor dimerization in the caveolae/lipid rafts enriched with cholesterol. Enhanced removal of cholesterol by AIBP/HDL from the EC plasma membrane disrupts caveolae/lipid rafts, resulting in retarded VEGFR2 signaling and restricted angiogenesis. AIBP: apoA-I binding protein; VEGFR: vascular endothelial growth factor receptor; HDL: high-density lipoprotein; EC endothelial cells.

associated membrane receptor activation, leading to inflammation and/or hyperproliferation and angiogenesis.^{2,64} This should be avoided in the plaque microenvironment, where intraplaque angiogenesis may incur vulnerability and culminate in its rupture.⁶⁵ It will be important to investigate if the enhancement effect of AIBP on cholesterol removal may improve the functionality of HDL in vivo and mitigate the development of atherosclerosis and CVD in at-risk patients.

AIBP documents a remarkable capability to restrict cell migration and angiogenesis in vitro in human ECs, ex vitro in mouse aortic rings, and in vivo in zebrafish.² This inhibitory effect of AIBP depends on cholesterol transporter ABCG1, which is responsible for cholesterol removal in ECs.⁶⁶ Accordingly, loss of ABCG1 abolishes the enhanced effect of AIBP on cholesterol efflux in ECs. Interestingly, neovascularization using mouse aortic ring assay demonstrates that ABCG1 deficiency increases microvessel sprouting, which is not retarded by AIBP. Consistently, the ability of AIBP to inhibit segmental artery angiogenesis is comprised in ABCA1- and ABCG11-deficient zebrafish. In this scenario, it is likely that there are increased levels of lipid rafts, similar to in myeloid cells,^{38,67} in ECs lacking ABCG1, which in turn promotes VEGFR2 signaling and thus augments angiogenesis. An in vivo assessment of lipid rafts in zebrafish, known as membrane lipid order, which was measured using a lipophilic probe laurdan, reveals that there are more lipid rafts in tip cells than in stalk cells. This is in line with other findings as tip cells, characteristic of VEGFR2 activation, are known to be responsible for vascular sprouting and migration in zebrafish. Stalk cells, instead, follow the migration of tip cells and are believed to be quiescent, although recent studies indicate that tip and stalk cells may compete and alternate their cell fates due to synchronization of VEGFR and Notch signaling pathways during angiogenesis.⁶⁸ The higher membrane order in tip cells can be lowered using cholesterol acceptor HDL, which has no effect on lipid raft abundance in the major blood vessel dorsal aorta (DA).² Since vasculogenesis, which gives rise to the DA, has completed at this time,⁶⁹ it is

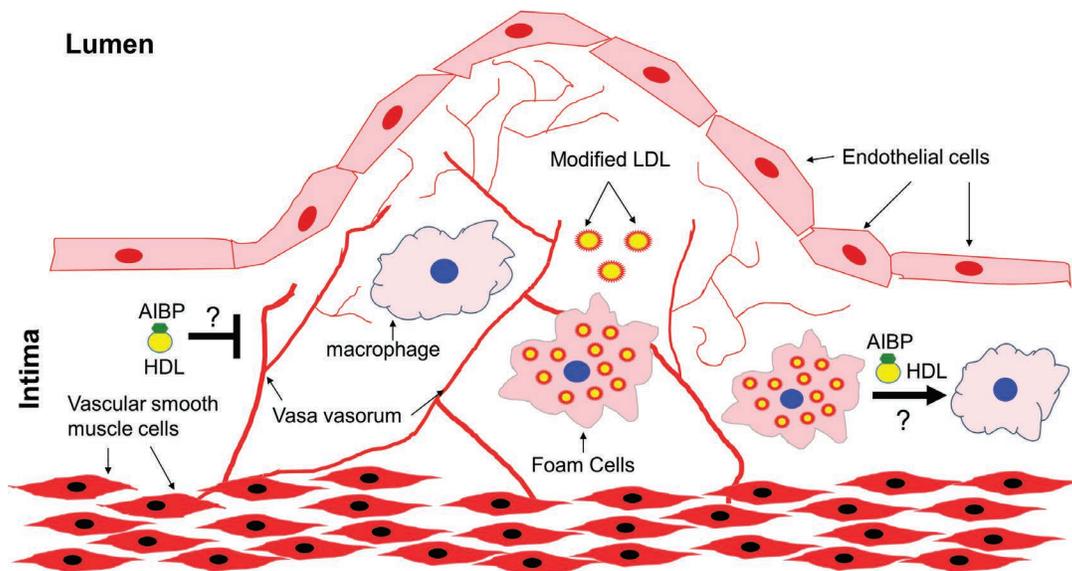


Figure 2. Presumptive protective role of AIBP in atherosclerosis. In atherosclerosis, LDL that is retained in the intima becomes proinflammatory modified LDL (e.g., OxLDL), which can be taken up by macrophages and become lipid-laden foam cells. AIBP, by accelerating cholesterol efflux, can reverse foam cell formation. In addition, AIBP may inhibit intraplaque angiogenesis and alleviate plaque growth. All these await further future studies. AIBP: apoA-I binding protein; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

comprehensible that the cells may be quiescent in this setting. This will be of particular importance in using AIBP to treat CVD because it manifests no adverse effect on established vessels. Rather, it only targets newly formed vasculature that is detrimental for atherogenesis.

Future Prospective: Using AIBP to Treat Human CAD

We found that downregulation of AIBP gene expression increases while upregulation decreases lipid rafts in the presence of HDL. Concurrent with this result, enhanced neovascularization is observed in the aortic rings from *ABCG1*^{-/-} mice.² Administering recombinant apoA-I or genetically raising levels of apoA-I inhibit angiogenesis and tumor growth in mice.⁷⁰ The blood vasculature and lymphatic vessels share many features and are functionally related. Lymphangiogenic sprouting initiates from venous ECs and intersegmental arteries provide cues for lymphangiogenesis.⁷¹ The potential role of AIBP in lymphangiogenesis may be worth investigating since lymphatics is regarded as a conduit for reverse cholesterol transport.

Considering that angiogenesis promotes atherosclerotic plaque growth and rupture, targeting AIBP may inhibit intraplaque angiogenesis and mitigate atherosclerotic plaque growth. Insights gained from zebrafish angiogenesis studies may be translated to targeting intraplaque neovascularization. We have made transgenic zebrafish with conditional expression of AIBP. Furthermore, taking advantage of the recently developed gene editing technology Crispr/Cas9,⁷² we can make an AIBP mutant zebrafish. Those AIBP loss- and gain-of-function zebrafish models will be of tremendous help in elucidating the role of AIBP in the early steps of atherogenesis. Inferred from studies of apoA-I mimetic peptides,⁷³ there may be AIBP derivate peptides or mimetics that can replace AIBP function and are more amenable for therapeutic applications.

Since AIBP also promotes cholesterol removal from cells, it is possible that AIBP could “kill two birds with one stone” by both inhibiting intraplaque angiogenesis and accelerating cholesterol efflux in the plaque settings (Figure 2). This may inhibit plaque growth and result in conversion of a vulnerable plaque to a stable plaque that poses less threat to patients. The therapeutic potential of AIBP to boost HDL functionality may also underpin the direction of future drug discovery for cardiovascular disease.

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Keywords: apoA-I binding protein, AIBP, angiogenesis, lymphangiogenesis, cholesterol efflux, reverse cholesterol transport

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