THE EMERGING ROLE OF ANGIOPOIETIN LIKE 8 (ANGPTL8) IN THE REGULATION OF TRIGLYCERIDE AND HDL-C LEVELS IN ATHEROGENIC DYSLIPIDEMIA

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Abstract

Coronary artery disease (CAD), a group of cardiovascular conditions that develops in response to atherosclerosis, is the most common cause of death worldwide. Atherogenic dyslipidemia, characterized by increased concentrations of circulating small-dense low-density lipoprotein cholesterol (LDL-C) and triglycerides, and decreased levels of high-density lipoprotein cholesterol (HDL-C), is a well-established determinant of heightened CAD risk. To date, several proteins have been found to contribute to the pathogenesis of dyslipidemia. Recently, angiopoietin like 8 (ANGPTL8), which belongs to a family of secreted proteins involved in lipid trafficking and metabolism, has emerged as a novel regulator of triglyceride and HDL-C levels. Here we provide an overview of ANGPTL8 regulation, function, and potential role in atherogenic dyslipidemia. We also present a summary of the evidence supporting genetic factors that contribute to the regulation of ANGPTL8 expression and function.

Keywords: ANGPTL8, dyslipidemia, high-density lipo-protein (HDL)-cholesterol, triglycerides, genetic variants, coronary artery disease.

1. Angiopoietin like proteins and dyslipidemia: a brief overview

Coronary artery disease (CAD) is the leading cause of death in the western world [1]. CAD develops in response to a limited flow of blood to myocardial cells, which commonly results from atherosclerosis. Recognized risk factors for CAD include family history, obesity, diabetes, hypertension, stress, sedentary lifestyle, and smoking. In addition, increased levels of serum low-density lipoprotein cholesterol (LDL-C) [2], lipoprotein (a) [3], and triglycerides (TG) [4] are linked to an elevated risk of developing CAD, while high levels of circulating high density lipoprotein cholesterol (HDL-C) provide a protective effect against disease [2]. Investigation of molecular mechanisms underlying the regulation of LDL-C, TG and HDL-C levels has been an active area of research for decades, and the relatively recent development of next generation sequencing (NGS) platforms has contributed significantly to our understanding of the role of genetic variation in maintaining blood lipid levels [5].

Angiopoietins are a family of vascular growth factors with a well-established role in angiogenesis and have been linked to diseases such as diabetes, sepsis, and cancer [6-9]. A family of secreted proteins that are structurally similar to angiopoietins, the angiopoietin-like proteins (ANGPTLs), have functional roles involved in inflammation [10], cancer cell invasion [11], hemapoietic stem cell activity [12], and lipid metabolism [13]. To date, eight members of this family have been identified: ANGPTL1 through ANGPTL8 [14-21]. All members contain an amino-terminal coiled-coil domain and a carboxy-terminal fibrinogen-like domain, with the exception of ANGPTL8, which lacks the fibrinogen-like domain [20]. In angiopoietin family members, this domain mediates binding to the TEK receptor tyrosine kinase (i.e., the angiopoietin receptor), which generates a signaling pathway leading to angiogenesis [22]. However, for ANGPTL proteins, or at least ANGPTL3, this domain binds to integrin $\alpha_v\beta_3$, leading to endothelial cell adhesion and migration and the induction of angiogenesis [23].

Since their discovery, ANGPTL proteins have been characterized as important mediators of circulating TG levels [24]. For example, ANGPTL3 and ANGPTL4 are recognized inhibitors of lipoprotein lipase (LPL), the rate-limiting enzyme for TG hydrolysis in circulating lipoproteins [25]. Deletion of ANGPTL3 or ANGPTL4 in mice is associated with decreased lipid levels [25-28]. Comprehensive reviews of the ANGPTL family members in the regulation of lipid metabolism can be found elsewhere [13, 29-31].

ANGPTL8 is the most recent addition to the ANGPTL family and shares 20% identity with the N-terminal domains of ANGPTL3 and ANGPTL4 [20]. In the short span of time since this classification, ANGPTL8, in conjunction with ANGPTL3, has emerged as an important inhibitor of LPL activity, with significant effects on triglyceride clearance [32]. For example, ANGPTL8 blockade in humanized ANGPTL8 mice resulted in decreased plasma TG levels and increased LPL activity [33]. Of interest, treatment of dyslipidemic cynomolgus monkeys with a single dose of high affinity monoclonal ANGPTL8 antibody normalized plasma TG levels and increased HDL-C levels by 30% [33]. Together, the cumulative literature suggests that inhibition of ANGPTL8 may represent a novel therapeutic approach for the treatment of dyslipidemia. The purpose of this review is to summarize the current literature supporting a role for ANGPTL8 in atherogenic dyslipidemia, the main cause of

atherosclerosis and CAD. We also discuss the effect of genetic variation in the ANGPTL8 locus on modulating levels of HDL-C in humans with atherogenic dyslipidemia.

2. ANGPTL8 is an important regulator of TG and HDL-C levels

The original annotation of ANGPTL8 appeared in Genbank in 2000, where it was termed hepatocellular carcinoma-associated protein TD26 (Dong X, Pang X, and Cheng W, unpublished work). However, the importance of ANGPTL8 in the liver was first revealed in a comprehensive transcriptome profiling study of 21 hepatocellular carcinoma cell lines to identify transcripts associated with expression of alpha-fetoprotein [34]. In that study, ANGPTL8 was not only expressed in hepatocellular carcinoma cell lines, but also correlated with alpha-fetoprotein levels. While ANGPTL8 was predicted to play a role in blood coagulation in this study, knockdown of this gene in mice resulted in hypotriglyceridemia in a subsequent report, suggesting an alternative role for this protein [35]. In 2012, a number of studies appeared in the literature, all of which firmly established a role for ANGPTL8 in lipid metabolism.

Using a combination of microarray analysis comparing transcript expression levels in 3T3-L1 preadipocytes with 3T3-L1 adipocytes 10 days post-differentiation and querying of publically available databases, ANGPTL8 was identified as a potential regulator of in vitro adipogenesis [36]. The previously unstudied transcript was predicted to encode a protein of 22 kDa and showed significant homology to ANGPTL3. Subsequent analysis of ANGPTL8 expression in 3T3-L1 cells during a seven-day process of in vitro adipogenesis showed a 30-fold upregulation in transcript levels at day three, which increased to 100-200-fold on days 4-7. These results were replicated during differentiation of murine and human primary preadipocyte cultures, in which ANGPTL8 expression increased up to 150-fold in human adipocytes compared with preadipocytes [36]. Both tumor necrosis factor-alpha (TNFα) and peroxisome proliferator-activated receptor gamma (PPARγ) are potent regulators of adipogenesis. Treatment of 3T3-L1 adipocytes and primary human adipocytes with TNFα resulted in significant downregulation of ANGPTL8 transcript levels. Knockdown of PPARγ in mature adipocytes corresponded with decreased ANGPTL8 transcript levels, suggesting that in these cells, ANGPTL8 is dependent on PPARγ [36]. ANGPTL8 transcript levels were also altered in the presence of insulin, isoprotenerol, forskolin, and dibutyryl-cAMP.

Assessment of ANGPTL8 in the ob/ob mouse model of type 2 diabetes showed elevated transcript levels in white adipose tissue and liver compared to wild type animals [36]. More strikingly, ANGPTL8 expression was upregulated 80-fold and 12-fold in white adipose tissue and liver, respectively, following refeeding of fasted mice. Because of these results, the authors renamed the previously annotated TD26 transcript, RIFL (refeeding-induced fat and liver). This comprehensive study was the first to highlight ANGPTL8 as a key player in adiogenesis and lipogenesis.

At approximately the same time, ANGPTL8 was also characterized as a nutritionally regulated factor, enriched in liver and playing a key role in lipid metabolism [37]. Using RNA-sequencing of liver and fat tissue obtained from mice following fasting or treatment with a high-fat diet, the authors identified ANGPTL8 as a novel gene regulated by nutritional state. Expression of ANGPTL8 in humans was found to be liver-specific, while in the mouse, expression was detected in liver and

both brown and white adipose tissues [37]. Like the previous study [36], hepatic expression of ANGPTL8 was found to increase in obesity and decrease with fasting. Due to its homology with regions in ANGPTL3 and ANGPTL4 that interact with lipoprotein lipase (LPL), the effect of ANGPTL8 on LPL was investigated. ANGPTL8 overexpression in mice was found to inhibit LPL activity, a finding that also correlated with increased TG levels in these animals. Thus, the main finding of this study was the presentation of a potential mechanism by which ANGPTL8 modulates TG levels by inhibiting LPL activity. Because of the connection between ANGPTL8 and LPL, the author named this factor lipasin [37].

In the same year, a third report emerged showing that ANGPTL8 was expressed in liver, adipose tissue, and adrenals of mice and circulated in the plasma of humans [20]. Expression of ANGPTL8 was reduced by fasting and increased by refeeding in mice, independent of sterol regulatory binding protein (SREBP)-1c, which is known to coordinate insulin-mediated lipogenic response in the liver. In humans, circulating ANGPTL8 levels were reduced following a 12-hour fast and consequently upregulated within three hours of refeeding [20]. Hepatic overexpression of ANGPTL8 in mice corresponded with increased plasma levels of TAG and nonesterified fatty acids, which were further elevated in the presence of ANGPTL3. Immunoprecipitation experiments showed that ANGPTL8 interacted with ANGPTL3. In cultured hepatocytes, ANGPTL8 expression correlated with the presence of N-terminal ANGPTL3 in the medium, suggesting that ANGPTL8 may promote cleavage of ANGPTL3. This finding was subsequently replicated in one study [38], but not another [39].

A database search for proteins related to the ANGPTL family revealed that ANGPTL8 was a paralog of ANGPTL3 [20]. ANGPTL8 was found to share ~20% identity with the N-terminal domains of ANGPTL3 and ANGPTL4 [20, 36]; however, ANGPTL8 terminates at residue 198 and thus lacks a C-terminal fibrinogen-related domain, as noted above. Of interest, ANGPTL8 and ANGPTL3 are located within introns of DOCK6 and DOCK7, respectively, suggesting that ANGPTL8 may have resulted from duplication of an ancestral DOCK gene [20, 36]. Because of the structural similarity with ANGPTL family members, the transcript was designated ANGPTL8, which presently remains the official name of this protein.

ANGPTL8 knockout mice were found to gain weight more slowly compared to wild-type animals, which appeared to be due to selective reduction in adipose tissue accretion [39]. In these animals, plasma TG levels were similar to wild-type mice in the fasted state, but decreased after refeeding and were associated with reduced VLDL-C secretion and increased post-heparin LPL activity in fed animals. Oral fat tolerance tests in ANGPTL8$^{-/-}$ mice showed increased clearance of plasma TG, and post-prandial increases in VLDL-TG update by adipose tissue were abolished in ANGPTL8$^{-/-}$ mice, suggesting that ANGPTL8 is required for trafficking of TG to peripheral tissues. Finally, ANGPTL8$^{-/-}$ mice showed no disruption in glucose homeostasis with either normal chow or high fat diet, indicating that metabolic changes that often accompany fat accumulation in adipose tissue do not alter glucose metabolism in these animals [39].

Although ANGPTL3 and ANGPTL8 both inhibit LPL activity and are known to interact with one another, up until recently, the manner in which they did so remained unclear. Experiments overexpressing

ANGPTL3 and ANGPTL8 in ANGPTL3$^{-/-}$, ANGPTL8$^{-/-}$, and wild-type mice were recently performed to address this question [32]. ANGPTL3 expression in ANGPTL8$^{-/-}$ mice corresponded with hypertriglyceridemia, while expression of ANGPTL8 in ANGPTL3$^{-/-}$ mice yielded no discernible effect on serum TG levels. Interestingly, while ANGPTL3 expression in ANGPTL3$^{-/-}$ mice corresponded with increased TG levels, this effect was exacerbated in the presence of ANGPTL8, suggesting that an interaction between the two proteins was required for ANGPTL8 to exert effects on TG levels. In HEK293 cells, which lack endogenous expression of ANGPTL3, ANGPTL8, and LPL, expression of ANGPTL8 alone failed to inhibit LPL. However, co-expression of ANGPTL3 with ANGPTL8, or expression of ANGPTL3 alone, resulted in strong inhibition of LPL activity, indicating that ANGPTL8 alone is not capable of inhibiting LPL, and can only do so in the presence of ANGPTL3. In this model, LPL inhibition produced by ANGPTL3 and ANGPTL8 was not blocked by an ANGPTL3 blocking antibody, suggesting the presence of an ANGPTL8 inhibitory motif. The authors used an anti-ANGPTL8 blocking antibody to demonstrate a reversal of ANGPTL8: ANGPTL3-mediated inhibition of LPL through steric obstruction of the ANGPTL8 inhibitory motif. Through this elegant set of experiments, the researchers showed that ANGPTL8 possesses a functional LPL inhibitory motif, and is only capable of inhibiting LPL and increasing plasma TG levels in the presence of ANGPTL3.

3. Association of regulatory and functional variants in ANGPTL8 with lipoprotein levels

A number of genome-wide association studies have reported evidence for genetic association between variants in the ANGPTL8 locus and HDL-C levels [40-46]. Evidence supporting regulatory or functional consequences of several of these variants has emerged, suggesting novel mechanisms by which genetic predisposition may contribute to variations in HDL-C levels.

Two reports identified rs737337 as the lead marker associated with HDL-C and total cholesterol, but not LDL-C or TG levels, in European cohorts [44, 47]. In an analysis conducted in Caucasian participants of the METabolic Syndrome in Men (METSIM) and African American participants from the Women's Health Initiative (WHI), rs737337 showed the strongest association with HDL-C levels [48]. This variant lies 2.8 kb upstream of the ANGPTL8 transcriptional start site and is situated in exon 19 of the dedicator of cytokinesis 6 (DOCK6) gene. This variant yielded allele-specific effects on ANGPTL8 expression in an assay for regulatory function [48]. Another marker, rs12979813, located 7.5 kb upstream of ANGPTL8 and in intron 22 of DOCK6, was associated with HDL-C levels, but not total cholesterol, in an African American population [40].

A third marker rs2278426, which creates an arginine to tryptophan substitution at position 59 of the ANGPTL8 amino acid sequence, has also been associated with concentrations of blood lipids. In Hispanic and African American members of the Dallas Heart Study, the variant T allele was associated with lower plasma levels of LDL-C and HDL-C [20]. Findings of association were replicated in African American participants of the Atherosclerosis Risk in Communities Study (ARIC) and the Dallas Biobank [20], and a recent GWAS for lipid traits in Mexicans also observed association between rs2278426 and HDL-C [45]. In Pima Indians and Mexican Americans, the variant T allele rs2278426 was associated with decreased levels of total cholesterol and HDL-C [38]. In individuals of European ancestry,

however, association findings have been less consistent. For example, in European American participants of ARIC, the variant allele was associated with significantly lower levels of HDL-C, but not LDL-C [20], while in an analysis using imputed genotypes from a genome-wide association study [44], the T allele was associated with levels of both HDL-C and LDL-C, although for the latter only at the nominal significance threshold [20]. In the Dallas Heart Study, rs2278426 was not associated with either HDL-C or LDL-C in European American participants [20].

Finally, rs145464906 was identified in a large-scale study of blood lipid levels in individuals of African and European ancestries. In that study, Caucasian, but not African American, carriers of the relatively rare (MAF 0.01-0.1%) variant allele had higher levels of HDL-C and lower TG levels, and a trend toward lower LDL-C was observed, but at levels that did not reach statistical significance [49]. Of interest, the variant allele predicts a premature stop codon, which would be expected to yield functional consequences for ANGPTL8.

Significant variability in allele frequency may reflect historical differences in selective pressures among various ethnic groups, which could account for the discrepancies in findings of association among populations with European ancestry. For example, the frequency of the variant T allele of rs2278426 in Caucasians is approximately 5%, which is far less common than it is in Mexican American (25%) and American Indian (50%) populations [38]. Similarly, the variant allele at rs145464906 described above was not observed in Pima Indians (unpublished data). Notably, the correlation between rs145464906 and rs2278426 was low and conditional analyses determined that the two variants are independent association signals [49], suggesting that some ANGPTL8 variants may have arisen in different ancestry groups.

A recent fine-mapping analysis of the ANGPTL8 promoter identified seven variants (rs12463177, rs17699089, rs200788077, rs56322906, rs3760782, rs737337, and rs3745683) showing allelic differences in transcriptional activity or protein binding [48]. While expression quantitative trait locus (eQTL) analysis using RNA from subcutaneous adipose tissue showed association only for marker rs4804154, the HDL-C-associated variants corresponded with ANGPTL8 and DOCK6 transcript levels [48]. However, only ANGPTL8 (but not DOCK6) transcript levels were associated with HDL-C levels in the METSIM samples and the variants most strongly associated with HDL-C levels were also more strongly associated with ANGPTL8 transcript levels, suggesting a regulatory locus at this gene [48]. In addition to these findings, rs12463177 increased ANGPTL8 transcriptional activity modestly (1.5-fold) and showed differential protein binding.

4. Conclusions

Despite its relatively recent discovery, ANGPTL8 has quickly gained prominence for its role in regulating TG levels through LPL inhibition. Through interaction with ANGPTL3 and with contribution from ANGPTL4, the ANGPTL3-4-8 model may represent a general framework by which TG trafficking is modulated [31]. Recent results showing restoration of TG levels and increased HDL-C in dyslipidemic monkeys using a fully human monoclonal ANGPTL8 provide strong evidence supporting ANGPTL8 as a potential target for the treatment of atherogenic dyslipidemia. Studies geared toward identifying molecular mechanisms underlying ANGPTL8 regulation and discovery of the cognate ANGPTL8

receptor are expected to provide even greater insight into the potential development of this protein as a therapeutic target for dyslipidemia.

Conflict of interest

The authors declare no conflict of interest

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Figure 1. Timeline of reports revealing a role for ANGPTL8 in lipid metabolism.