The Paraoxonase Gene Family and Atherosclerosis

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Management of serum low-density lipoprotein has been a cornerstone of cardiovascular medicine for the past two decades. More recently, the attention paid to the protective role of high-density lipoprotein (HDL) in atherosclerosis has increased substantially, particularly with respect to the antioxidant properties of HDL. Considerable evidence supports the notion that the paraoxonase gene family is largely responsible for the antioxidant properties of HDL. This article reviews the three known members of the paraoxonase family and the evidence that supports their likely role in the development and progression of atherosclerosis.

Introduction

Atherosclerosis, particularly coronary artery disease and cerebrovascular accident, remains the leading cause of mortality in the industrialized world [1]. Over the past two decades, management of serum cholesterol has become one of the established tenants in the treatment of atherosclerosis. Much of the attention has been paid to the use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors to reduce low-density lipoprotein (LDL). However, role of high-density lipoprotein (HDL) is equally, if not more, important in the pathogenesis of atherosclerosis. Levels of HDL are inversely related to the development of cardiovascular atherosclerosis [2]. Every 1-mg/dL increase in HDL is associated with a 2% to 3% decrease in adverse cardiovascular events [3]. There is substantial evidence suggesting that levels of HDL may be more predictive of future cardiovascular events than LDL. In one study of 13,000 people, Sacks et al. [4] found that low serum HDL was a stronger predictor of future adverse cardiovascular events than elevated LDL levels. In another study of patients who already had low or near normal LDL levels, levels of serum HDL were still strongly predictive for coronary heart disease [5]. Other studies strongly suggest that high levels of serum HDL cholesterol may actually have a direct protective effect [6]. Individuals with elevated HDL levels of 75 mg/dL or higher have significant longevity and are relatively free from atherosclerosis.

There are two proposed mechanisms through which HDL is believed to exert its antiatherogenic effects. The first is the process of reverse cholesterol transport, through which free cholesterol produced by cells is transferred by HDL molecules to the liver [3,7]. It is believed that HDL particles also remove cholesterol from macrophages that reside within atherosclerotic plaques through this process. Researchers have sought to harness reverse cholesterol transport as a means of treating atherosclerosis. One strategy has been to directly infuse a synthetic variant of apolipoprotein (apo) A-I, the major structural component of HDL, into both animals and humans [8–10]. The apoA-I Milano molecule was first identified in a population of Italian individuals with low levels of serum HDL but paradoxically little clinical atherosclerosis [11]. It is believed that direct infusion of the recombinant apoA-I Milano molecule led to a direct reduction in atherosclerotic plaque burden via reverse cholesterol transport. Another strategy has been to inhibit the enzyme lecithin-cholesterol acyltransferase (LCAT) and thereby push the reverse cholesterol transport mechanism toward transferring cholesterol to the liver rather than LDL particles. The results of clinical trials using LCAT inhibitors showed a modest reduction in atherosclerosis but also an increase in adverse cardiovascular events [12••,13••,14].

The second proposed mechanism through which HDL exerts its atherogenic effects is believed to be by reducing systemic and local inflammation. The pathophysiology for the development and progression of atherosclerosis begins with and is perpetuated by the entry of LDL particles into the arterial vessel wall through disruptions in the endothelium [15]. Once the LDL particles are inside the vessel wall, they undergo oxidation by processes such as the lipoxygenase and myeloperoxidase pathways [16]. The oxidized LDL particles trigger the synthesis and release of factors such as monocyte chemotactic protein-1 and

adhesion molecules that precipitate the recruitment and migration of inflammatory cells to the arterial wall. This perpetuates a feed-forward cycle of continued LDL oxidation, inflammatory cell recruitment, and inflammation [15]. HDL appears to circumvent this cycle of inflammation by protecting LDL from oxidation and by abrogating the recruitment and migration of inflammatory cells to the arterial wall [7,17–19]. The ability of HDL to reduce LDL oxidation has been shown a number of times in different experimental in vitro models of LDL oxidation [16,20]. In each of these studies, LDL oxidation was significantly reduced when incubated together with HDL molecules versus when LDL was incubated alone. HDL causes the enzymatic hydrolysis of oxidized phospholipid molecules of LDL particles, and the modified phospholipid particles are transferred to the HDL molecules. Reducing the levels of oxidized LDL therefore blunts the normal immune response to oxidized LDL [21]. There may also be other effects of preventing LDL oxidation, such as reducing endothelial cell death [22] and blocking macrophage foam cell formation [16]. The antiatherogenic and antioxidant effects of HDL have been studied extensively. There is substantial evidence that the antioxidant properties of HDL are attributed to the paraoxonase gene family.

Paraoxonase Gene Family

There are three known members of the paraoxonase gene family: paraoxonase 1, paraoxonase 2, and paraoxonase 3 [23]. Paraoxonase 1 (*PON1*) was the first identified member and was characterized for its role in the binding and hydrolysis of organophosphates [24]. PON1 has a substantial role in protecting the nervous system from insecticides and various nerve agents. PON1 was named for its ability to metabolize the primary metabolite of the insecticide parathion, which is paraoxon. Incidentally, none of the other members of the paraoxonase family appear to have any significant ability to metabolize organophosphates [25,26]. Researchers first became interested in a possible role of PON1 in cardiovascular disease after learning that PON1, after synthesis and secretion by the liver, is exclusively associated with HDL in the serum [23,27]. PON1 was found to catalyze the breakdown of phospholipid hydroperoxides [24]. In vivo, this activity would normally generate potentially damaging lysolipids, aldehydes, and ketones. However, HDL has shown to be able to safely sequester these compounds [18,20]. This close association between PON1 and HDL as well as the enzymatic activity has prompted close scrutiny of the potential role of *PON1* in cardiovascular atherosclerosis.

Many studies have shown that in experimental in vitro models of LDL oxidation, usually by co-incubation with metals such as copper, purified PON1 blocks LDL oxidation [20,26,28]. Other studies have shown that PON1 also blocks HDL oxidation and, consequently, perpetuates HDL's antioxidant activity [29]. Furthermore, in vitro studies of PON1 have shown that by blocking LDL oxidation, purified PON1 also blocks the release of chemotactic factors and therefore the migration of inflammatory cells [20,26,28].

In vivo animal studies using mouse models have also supported the antioxidant activity of PON1. In mice and rabbits, *Pon1* gene expression in the liver decreases when they are fed a high-fat atherogenic diet [19,30,31]. In the *ApoE* knockout (*ApoE*$^{-/-}$) mouse (a standard model for atherosclerosis), a concurrent homozygous *Pon1* knockout leads to a substantial increase in the development of atherosclerosis relative to *ApoE*$^{-/-}$ mice with one or both copies of *Pon1* [32]. The LDL particles from mice with the double *ApoE Pon1* knockout contained significantly higher levels of oxidized phospholipids [33]. Furthermore, HDL isolated from the *Pon1* knockout mice was unable to block the accumulation of lipid peroxides by LDL particles in vitro.

HDL from mice that overexpress *Pon1* shows an enhanced ability to block LDL oxidation and lipid peroxide accumulation in vitro [34]. If these same mice are fed a high-fat diet with cholic acid, *Pon1* overexpression blocks the development of fatty streak formation in the aortic root compared with wild-type mice [35]. In ApoE$^{-/-}$ mice that overexpress *Pon1*, the higher *Pon1* levels lead to a significant reduction in atherosclerotic lesion formation relative to ApoE$^{-/-}$ mice with normal *Pon1* levels.

Direct evidence of PON1 activity in the context of cardiovascular disease in humans has been more limited. One study found an inverse relationship between HDL PON1 activity and LDL oxidation levels in humans. In humans, smoking and ingestion of trans fats causes a reduction in PON1 activity [36,37,38]. In contrast, moderate alcohol consumption, vitamins C and E, and polyphenols increase PON1 activity [39]. Simvastatin also appears to enhance PON1 activity and may be one of the pleiotropic effects that contribute to the antiatherogenic properties of these medications [40].

Both *PON2* and *PON3* were identified subsequently to *PON1*, and less is known about these members of the paraoxonase family. PON2 is an intracellular protein that is widely expressed across a number of tissues [41]. Physiologically, PON2 shows a great deal of similarity to PON1. In experimental in vitro oxidation models, such as exposure to free metals or incubation with cultured endothelial cells, LDL incubated together with purified PON2 protein showed significantly less oxidation relative to LDL incubated alone [25,33]. In fact, in these same experimental oxidation models, if the cells overexpress *PON2*, there is also a significant reduction in LDL oxidation. Also, if cells overexpressing *PON2* are incubated with mildly oxidized LDL, the LDL can revert to a nonoxidized state.

Unlike PON1, PON2 is not present on HDL molecules. PON2 may act instead at the cellular level. Cells

that overexpress *PON2* show significantly lower intracellular oxidative stress following exposure to hydrogen peroxide or oxidized phospholipids compared with normal cells [25]. Researchers have found that when mouse macrophages are exposed to oxidative stress, there is an increase in *Pon2* expression and activity [42]. Both wild-type mice and *ApoE*$^{-/-}$ mice fed a high-fat diet exhibit significantly higher levels of *Pon2* in various tissues compared with mice fed a regular chow diet. Similarly, monocytes harvested from humans with hyperlipidemia also show significantly higher levels of *Pon2* compared with individuals with normal cholesterol levels [43].

PON3, like PON1, has been shown to be associated with HDL particles but at significantly lower levels, at least in mice. Like the other members of the paraoxonase gene family, PON3 has significant antioxidant activity [26]. Experimentally, purified PON3 inhibits the oxidation of LDL when they are co-incubated in vitro [44]. When cells overexpressing PON3 are placed in culture with LDL, the LDL particles accumulate significantly fewer lipid peroxides [26]. As with PON1 and PON2, the ability of PON3 to reduce the modification of LDL leads to a subsequent reduction in monocyte chemotaxis.

As with PON2, in vivo studies of the functional role of PON3 in atherosclerosis are ongoing. But we can infer that PON3 activity alone in mice is not sufficient to prevent atherosclerosis. In *ApoE*$^{-/-}$ mice null for *Pon1* but with preserved *Pon3* activity, atherosclerosis continues unabated [32,33]. This could be related to the lower levels of *Pon3* in mouse HDL and may not be relevant to human disease because human HDL contains significantly higher levels of *Pon3* relative to mice.

In our genomic studies of atherosclerosis using mouse models, we have also found that *Pon2* expression is significantly associated with atherosclerosis. In one study, we looked for genes associated with atherosclerosis in the context of different risk factors, such as age, diet, genotype, and gender. For example, we sought to define the genes associated with atherosclerosis in the setting of a high-fat diet as opposed to a normal diet, or genes associated with atherosclerosis in the setting of a combination of risk factors, such as age and diet. The raw data showed that *Pon2* expression in the aortas of *ApoE*$^{-/-}$ mice 6 weeks of age that were fed a chow diet was significantly higher than in *ApoE*$^{-/-}$ mice 12 weeks of age fed a high-fat diet. This finding is somewhat in contrast with the other research findings that indicate high-fat diets increase PON2 expression. However, in the context of aortic atherosclerosis, we found that *Pon2* expression was significantly lower. In our study, we found that the *Pon2* was a key gene associated with atherosclerosis in the context of diet (ie, *Pon2* may be contributing to the development of atherosclerosis in the context of diet as a risk factor) [45] (Seo and Goldschmidt-Clermont, unpublished data).

Paraoxonase Gene Family: Genetics and Atherosclerosis

Given the preponderance of evidence supporting the potential role of the paraoxonase gene family in atherogenesis, substantial research has looked for a genetic association between paraoxonase gene variants and atherosclerosis.

There are two well-studied polymorphisms in *PON1*: 1) a glutamine to arginine substitution at codon 192 (Gln192Arg) and 2) a methionine to leucine substitution at codon 55 (Met55Leu) [46]. There are also five known polymorphisms located within the *PON1* promoter region that have been shown to affect *PON1* expression, but they have not yet been conclusively associated with human atherosclerosis [47–50].

The Gln192Arg polymorphism is associated with the development of stroke and coronary artery disease. In addition, this polymorphism has been shown to be associated with type 2 diabetes mellitus [51] and Parkinson's disease [52,53]. *PON1* with a glutamine at the 192 position has been demonstrated to be more effective in preventing LDL oxidation [54]. Over 40 genetic association studies in a number of different racial and ethnic populations have shown a significant association between the arginine for glutamine substitution and adverse cardiovascular outcomes [55]. There is some evidence to suggest that the arginine substitution may affect *PON1* activity, but it may also increase susceptibility to other established risk factors of atherosclerosis such as diabetes mellitus and smoking.

The Met55Leu polymorphism has shown statistical association with coronary artery disease and cerebrovascular disease in the literature. There also appears to be a strong association between the Met55Leu polymorphism and Parkinson's disease [56]. *PON1* with methionine at the codon 55 position is associated with enhanced protection against LDL oxidation [54]. Incidentally, this polymorphism has been shown functionally to explain some of the variations in the ability of *PON1* to metabolize organophosphates [57]. The evidence for the genetic association with coronary artery disease is not as strong for the Met55Leu polymorphism, with studies showing a positive association as well as no association [55].

A number of polymorphisms have been identified in *PON2*. Of these, the most compelling evidence for genetic association has been the serine for cysteine substitution at codon 311 (C311S) [58,59]. Sanghera et al. [59] looked at the Cys311Ser polymorphism in a population of Asian Indians and found a significant association between the serine for cysteine substitution and coronary artery disease. Compared with the CC311 genotype, presence of the CS311 or SS311 genotype was associated with an adjusted odds ratio for developing coronary artery disease of 2.5, but this was a small study with only 129 participants. In a larger study by Chen et al. [60], the Cys311Ser polymorphism was studied in 711 participants from the

Women's Ischemia Syndrome Evaluation (WISE) study. In this study, researchers found no association between this polymorphism and coronary artery stenosis severity. However, they did find that the frequency of the Cys311Ser polymorphism was significantly higher in individuals with three-vessel coronary artery disease.

Much less is known about the association between the genetics of *PON3* and atherosclerosis. In sequencing a group of 250 individuals from southern Italy, researchers found five point mutations in *PON3* [61]. Of these five, three were silent and two were missense mutations. No studies looking for a specific association between *PON3* variants and atherosclerosis have been performed, although one study has proposed that *PON3* genetic variation may influence atherosclerosis by modulating *PON1* activity [62].

Several genome-wide association studies (GWAS) to identify polymorphisms associated with coronary atherosclerosis or myocardial infarction have been reported [63–65]. The genetic association studies for the paraoxonase gene family described previously examine specific polymorphisms for their association with cardiovascular disease. GWAS are conducted without targeting a specific polymorphism. Instead, these studies perform a massive number of genotypes (300,000 to 1 million) on a large number of participants (> 1000). This enormous data generation is followed by an analysis to identify polymorphisms that are associated with a disease or disease outcome. In the GWAS studies of coronary artery disease or myocardial infarction that have been reported, none have identified polymorphisms within the paraoxonase gene family.

Conclusions

Over the past decade, the treatment of elevated LDL has been a cornerstone in the management of atherosclerotic cardiovascular disease. The next stage of lipid management will focus attention not just on serum HDL levels, but also on augmenting the beneficial functions of HDL (eg, boosting reverse cholesterol transport and antioxidant activity). The paraoxonase gene family appears to be a key contributor to HDL's antioxidant activity. From a therapeutic perspective, all three members have demonstrated the ability to prevent LDL oxidation and block, at least to some extent, the migration of inflammatory cells. If these laboratory findings can be translated into clinical applications, we may have a truly powerful means of blocking atherosclerosis development and progression. The tendency of free serum PON1 to be avidly taken up by HDL and consequently boost antioxidant activity by HDL could be exploited for therapeutic purposes if we can find a means to deliver PON1 to the circulation.

From a diagnostic and prognostic perspective, substantial research efforts have started to identify and confirm genetic polymorphisms in the members of the paraoxonase gene family that are associated with coronary artery disease, cerebrovascular disease, myocardial infarction, and stroke. These genetic markers would likely provide much more precise information that would go beyond measurements of serum HDL levels and provide information about HDL activity. Knowledge of robust genetic markers would be valuable for identifying individuals prior to disease initiation or in the early stages of atherosclerosis development and would permit aggressive preventive therapies. The most validated marker is the Gln192Arg polymorphism of *PON1,* which is associated with coronary artery disease and has a functional impact on enzymatic efficacy. Clearly, more studies will be needed for further validation of the association of Gln192Arg and other genetic polymorphisms in the paraoxonase gene family with atherosclerosis. Further study is also required to understand the diagnostic and prognostic implications of the validated genetic markers in the context of clinical risk factors such as diet, exercise, and tobacco use.

The paraoxonase gene family represents a potentially important target for therapeutic interventions and diagnostic/prognostic information. Substantial research efforts are under way involving animal models and humans to obtain more detailed and comprehensive information about the paraoxonase gene family to allow us to translate this knowledge to clinical applications.

Disclosure

No potential conflicts of interest relevant to this article were reported.

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