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Commentaries

Preclinical Reversal of Atherosclerosis by FDA-Approved Compound that Transforms Cholesterol into an Anti-Inflammatory "Prodrug"

Andrew R. Mendelsohn[1,2] and James W. Larrick[1,2]

Abstract

Although atherosclerosis is treatable with lipid-lowering drugs, not all patients respond. Hydroxypropyl-beta-cyclodextrin (CD) is an FDA-approved compound for solubilizing, capturing, and delivering lipophilic drugs in humans. Zimmer et al. report that CD mediates regression of atherosclerotic plaques in two mouse models by solubilizing cholesterol crystals (CCs), and promoting metabolism of CCs into water-soluble 27-hydroxycholesterol, which, in turn, activates anti-inflammatory LXR receptor target genes, promotes active and passive efflux of cholesterol from macrophages, and increases metabolic processing of cholesterol. In effect, CD inverts the role of its cargo, cholesterol, from inflammatory to anti-inflammatory by converting cholesterol into a ''prodrug'' that when modified to 27-hydroxycholesterol reduces atherosclerosis. This mechanism defines a new class of pharmaceuticals, ''inverters'': compounds that cause innate biomolecules to act opposite to their normal function. However, chronic CD treatment in animal models damages auditory cells, which must be addressed before CD can be developed as a systemic drug for atherosclerosis.

Introduction

Atherosclerosis is a disease of arterial wall remodeling, in which lipids accumulate in the subendothelial layer, leading to an inflammatory response that stimulates further pathogenic changes.[1] Atherosclerosis underlies cardiovascular disease, leading to heart attacks and strokes, which are common causes of death. Atherosclerosis is a progressive disease that is often associated with aging.

In atherosclerosis, lipid deposition leads to the formation of cholesterol crystals (CCs), which stimulate inflammation by inducing innate immunity pathways, complement activation, neutrophil extracellular trap formation, and transformation of macrophages into foam cells. Targeting CC formation has been a successful therapeutic approach with lipid-lowering drugs considered a first-line treatment to block progression of atherosclerosis. Although existing lipid-lowering drugs that target low density lipoprotein (LDL) levels can slow or sometimes halt progression of atherosclerosis, they often do not reverse the pathogenesis of atherosclerosis and are ineffective in some patients.[2–4]

One simple idea is to attempt to solubilize cholesterol in atherosclerotic plaques to promote its clearance and catabolism. A possible agent to solubilize cholesterol is 2-hydroxypropyl-beta-cyclodextrin (CD), an FDA-approved compound for solubilizing, capturing, and delivering lipophilic drugs in humans.[5,6] Preliminary reports suggest that in cultured cells, CD can solubilize cholesterol, increasing solubility in aqueous solution 150,000×, stimulate cholesterol efflux from foam cells, and reduce inflammation.[7,8]

Cholesterol-Solubilizing Compound Reverses Atherosclerosis

In a potentially groundbreaking preclinical study, Zimmer et al. show that CD treatment inhibits and partially reverses atherosclerosis in several mouse models of atherosclerosis.[9] In an initial experiment, mice deficient in apolipoprotein E ($ApoE^{-/-}$) were fed a cholesterol-rich diet simultaneously with twice weekly subcutaneous injections of CD for 8 weeks. CD reduced plaque area relative to untreated animals (60%–35%) and CC density (0.28–0.18) in the aortic root, as well as greater than 50% reductions in reactive oxygen species, and inflammatory cytokines TNF-alpha, IL-6, and IL-8. Beyond the change in CCs, plaque composition was unchanged as were serum cholesterol levels. General physiological parameters such as weight and blood pressure were also unchanged.[9] This experiment demonstrated that CD could inhibit atherosclerosis.

To determine whether CD could actually reverse atherosclerosis, $ApoE^{-/-}$ mice were fed a cholesterol-rich diet for 8 weeks to induce robust plaque formation and then switched

[1]Panorama Research Institute, Sunnyvale, California.
[2]Regenerative Sciences Institute, Sunnyvale, California.

to a normal chow diet or maintained on the cholesterol-rich diet. CD treatment resulted in a 45% regression of plaque size (to values similar to that seen in the simultaneous dosing study) for both diets, suggesting that *CD can regress pre-existing plaques* to a similar extent that it can prevent plaque formation. Furthermore, CC formation was reduced to similar levels seen for simultaneous CD treatment for mice continuing on the cholesterol-rich diet and to almost 50% lower levels when switched to the normal chow, suggesting that the beneficial effect of CD can be enhanced by lowering lipids, which might imply potential synergy with pre-existing drugs such as statins that lower cholesterol. These results were confirmed in the LDL-$R^{-/-}$ mouse model of atherosclerosis as well (see hereunder).[9]

As expected, CD increased solubility of CCs *in vitro*, where 10 mM CD was capable of almost completely solubilizing 1 mg of CCs. At a dose of 2 g/kg, and assuming a 25 g mouse has 1.6 mL of blood, the maximum blood concentration of CD in these animals is about 30 mM. In contrast, CD probably does not achieve the maximum calculated concentration in the blood and it will bind other lipids as well, but the high CD dosage used in Zimmer et al.'s experiments could potentially solubilize deposited CCs. Because CCs are often intracellular, Zimmer et al. treated macrophages in culture with fluorescently labeled cholesterol and then determined that 10 mM CD could dissolve 50% of the intracellular CCs.[9]

To determine whether the observed antiatherosclerotic effects went beyond mere solubilization of CCs and involved increased metabolism of cholesterol, Zimmer et al. loaded macrophages with CCs composed of cholesterol labeled with six deuterium atoms (D6-cholesterol) so that the metabolites could be identified by gas chromatography–mass spectrometry-selective ion monitoring (GC-MS-SIM). A significant amount of cholesterol esters was observed after CD treatment. Cholesterol esters, formed by the action of acetyl CoA acetyltransferase (ACAT1), are less cytotoxic than cholesterol and are typically stored in lipid droplets. Also, the total cellular pool of D6-cholesterol was reduced by efflux of the labeled cholesterol to the medium. It is likely that active transport through the ABCA1 and ABCG1 reverse cholesterol transport (RCT) is involved, given that CD increased ABCA1 and ABCG1 expression beyond the levels associated with CCs treatment.[9] However, the importance of active transport was not clearly established in this experiment.

ABCA1 and ABCG1 transporters are positively regulated by oxysterols through LXR/RXR receptors. Zimmer et al. determined that production of 27-hydroxycholesterol, a freely diffusible oxysterol, was increased 15-fold by CD in CC-treated macrophages. Interestingly, CYP27a1 the enzyme that catalyzes the formation and secretion of 27-hydroxycholesterol was not upregulated. Furthermore, 27-hydroxycholesterol levels increased even in control macrophages grown in medium with normal levels of cholesterol.[9] Apparently, increased levels of 27-hydroxycholesterol are due to the solubilization of CCs into free cholesterol so that it is available as a substrate for CYP27a1.

Consistent with the activation of LXR genes, genome-wide mRNA profiling indicated that LXR target gene expression,[10,11] including ABCA1 and ABCG1, was increased by CCs and even further increased by CD after CCs. Moreover, CD was sufficient to induce LXR target gene expression even without prior CC treatment. As expected, expression of genes known to be downregulated by LXR activation, such as proinflammatory cytokines IL-1B, IL6, and TNF-alpha, was reduced. To prove the point, macrophages engineered to lack LXR function (LXRa-/LXRb-) do not induce LXR target gene expression under any of these treatments.[9] These results are not entirely unexpected as previous work has shown that CD can not only mobilize cholesterol that is trapped in the lysosomes by a mutation in the NPC1 transporter in Niemann–Pick Disease Type C (NPC) but also activate LXR-regulated genes.[11,12]

To test whether CD stimulated active transport of CCs *in vivo*, bone marrow-derived macrophages from wild-type (WT) or LXRa/LXRb mice were loaded with D6-CCs *in vitro* and then injected into the peritoneum of WT mice. CD stimulated the excretion of D6-cholesterol into the feces, which suggests the involvement of active transport, and into the urine, which suggests that CD also stimulates passive transport through cholesterol solubilization. These results were extended to humans in an experiment with three NPC patients. Intravenous injection of CD resulted in increased urinary cholesterol excretion over 10 hours, suggesting CD solubilizes cholesterol in humans and that CD-mediated passive cholesterol efflux occurs in both mice and humans.[9]

To further determine whether CD might also reverse atherosclerosis in humans, human carotid artery biopsies containing plaques were cultured for 24 hours with or without CD. Again CD promoted the transfer of cholesterol from plaques to supernatant and the production of LXR activator 27-hydroxycholesterol. Simultaneous global gene expression experiments were similar to those from the mice experiments: LXR-regulated target genes were increased and genes involved in regulation of inflammatory response were decreased, including the inflammasome sensor NLRP3, as was observed for the mice.[9]

Using the LDL-R–/– mouse model of atherosclerosis, Zimmer et al. confirmed that macrophages possessing functional LXR are necessary for atherosclerotic plaque regression by irradiating the mice to destroy their immune systems and then transplanting bone marrow from LXRa/LXRb or WT mice to reconstitute their immune systems. Unlike using transplants from WT mice, no plaque reduction was found in mice with macrophages lacking LXR. Interestingly, similar experiments with macrophages lacking the ABCA1 and ABCG1 transporters *did* show plaque reduction, which suggests that active transport RCT is not necessary for plaque regression.[9] One possible explanation is that the anti-inflammatory effects promoted by CD perhaps through 27-hydroxycholesterol synthesis are of paramount importance.

The mechanism of CD in atherosclerosis is novel. CD interacts with cholesterol and CCs in such a way as to *invert* their role from promoting to inhibiting inflammation. Increasing cholesterol's enzymatic conversion to 27-hydroxycholesterol essentially makes the cholesterol/CD complex a ''prodrug.'' *CD defines a new kind of pharmaceutical, an ''inverter'': a compound that causes innate biomolecules to act inversely to their function*. This is possible because there is a feedback mechanism that controls the extent to which cholesterol increases inflammation.

The proinflammatory signaling associated with excess cholesterol or CCs induces an anti-inflammatory braking mechanism through modification to 27-hydroxycholesterol. Pathology arises when too much cholesterol is present in CCs and can be reversed by increasing the amount of 27-hydroxycholesterol.

Medical Implications

These preliminary studies suggest that CD may be a useful therapeutic to potentially reverse atherosclerosis. However, there are caveats. The mouse models used in these studies are imperfect.[13] For example, ApoE mice can be manipulated to show significant plaque reduction by feeding a reduced cholesterol diet. In humans, it is typically more difficult to reverse atherosclerosis, although dietary changes and statin drug therapy are often sufficient to halt or slow progression. Certainly the CD studies would have to be replicated and then appropriate randomized clinical trials performed.

A key issue may be unwanted side effects. Even though CD (a) is considered safe, (b) has a very high LD50, (c) is commonly used in drug formulations, and (d) is in clinical trials for NPC1 (NCT02534844), the compound is not without problems. Chronic treatment of NPC1 cats with CD significantly extends lifespan, inhibits neurological symptoms, and blocks cerebellar dysfunction and Purkinje cell death. However, these benefits are accompanied by hearing loss[14] by removing cholesterol from prestin in sensory cells.[15] This problem may have solutions. Should CD be developed for treating atherosclerosis in humans, perhaps treatment could be intermittent to limit or prevent irreversible damage to hearing. In that scenario, after regression of plaques, lipid-lowering therapies could be employed to maintain healthy arteries. Alternatively, perhaps it might be possible to use cholesterol-containing ear drops to supplement the sensory hairs sufficiently to afford protection.

Questions remain with regard to viability of commercial development of CD. Although Vitesse, Inc. may have been granted orphan drug status for CD to treat NPC1, atherosclerosis is not an orphan disease and CD is probably not patentable for this application, making development through a traditional pharmaceutical model difficult. Coupled with the potential damage to the auditory system, it is very unlikely that CD will be developed for atherosclerosis unless a government or nonprofit organization with deep pockets steps in.

One obvious question arising from this work is why not just target LXR, since Zimmer et al. and earlier studies point to this potentially very effective drug target for atherosclerosis? The answer is that LXR has been targeted. LXR agonists do show significant benefit in preclinical studies,[16,17] but these drugs showed liver toxicity and lipogenic effects,[18,19] unlike CD.

Conclusion

The idea of melting away atherosclerotic plaques is an appealing one. CD, considered generally safe by the FDA, is a relatively benign substance representing a potentially significant and inexpensive molecule to treat atherosclerosis. However, beyond confirmatory preclinical studies, which are likely to succeed, the potential chronic side effects on hearing need to be addressed.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Andrew R. Mendelsohn
Regenerative Sciences Institute
and Panorama Research Institute
1230 Bordeaux Drive
Sunnyvale, CA 94089

E-mail: amend@regensci.org