Thematic review series: The Pathogenesis of Atherosclerosis

The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL

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Abstract   For more than two decades, there has been continuing evidence of lipid oxidation playing a central role in atherogenesis. The oxidation hypothesis of atherogenesis has evolved to focus on specific proinflammatory oxidized phospholipids that result from the oxidation of LDL phospholipids containing arachidonic acid and that are recognized by the innate immune system in animals and humans. These oxidized phospholipids are largely generated by potent oxidants produced by the lipoxygenase and myeloperoxidase pathways. The failure of antioxidant vitamins to influence clinical outcomes may have many explanations, including the inability of vitamin E to prevent the formation of these oxidized phospholipids and other lipid oxidation products of the myeloperoxidase pathway. Preliminary data suggest that the oxidation hypothesis of atherogenesis and the reverse cholesterol transport hypothesis of atherogenesis may have a common biological basis. The levels of specific oxidized lipids in plasma and lipoproteins, the levels of antibodies to these lipids, and the inflammatory/anti-inflammatory properties of HDL may be useful markers of susceptibility to atherogenesis. Apolipoprotein A-I (apoA-I) and apoA-I mimetic peptides may both promote a reduction in oxidized lipids and enhance reverse cholesterol transport and therefore may have therapeutic potential.—Navab, M., G. M. Ananthramaiah, S. T. Reddy, B. J. Van Lenten, B. J. Ansell, G. C. Fonarow, K. Vahabzadeh, S. Hama, G. Hough, N. Kamranpour, J. A. Berliner, A. J. Lusis, and A. M. Fogelman. **The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL.** *J. Lipid Res.* 2004. **45: 993–1007.**

LDL OXIDATION AND ARTERY WALL CELL CYTOTOXICITY

In 1979, Chisolm and colleagues (1) reported that the oxidation of LDL was injurious to artery wall cells and suggested that LDL oxidation may be important in atherogenesis. They also demonstrated that HDL inhibited the LDL-induced cytotoxicity (1). Over the ensuing two decades, this group elucidated the basis for these observations and established the important role of oxidized cholesterol products, especially cholesterol hydroperoxides (2).

THE SEARCH FOR MECHANISMS OF LDL-INDUCED FOAM CELL FORMATION

Also in 1979, Goldstein et al. (3) reported that acetylated LDL but not native LDL was taken up by "scavenger receptors" instead of the LDL receptor, resulting in cholesteryl ester accumulation in macrophages characteristic of foam cells. Because acetylation was not known to occur, after the publication of this seminal paper there was a search for physiological ligands that would explain foam cell formation. Fogelman et al. (4) soon reported that malondialdehyde, an obligate product of the oxidation of arachidonic acid by the lipoxygenase pathways, could cause Schiff-base formation with the epsilon amino groups of apolipoprotein B (apoB) lysines in LDL. The altered lipoprotein was recognized by macrophage scavenger receptors, resulting in cholesteryl ester accumulation characteristic of foam cells. The next year, Steinberg and colleagues (5) demonstrated that cultured endothelial

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cells in a medium rich in metal ions and in the absence of the antioxidants present in serum could oxidize LDL to a form recognized by macrophage scavenger receptors. Three groups reported on the mechanism of this modification of LDL by artery wall cells in 1984 (6–8). In 1988 and 1989, papers were published indicating that oxidatively modified LDL was present in the artery walls of animals and humans with atherosclerosis (9–11).

Witztum, Steinberg, and Parthasarathy (12–15) and Chisolm (16) emphasized that the cells of the artery wall transfer oxidative waste products into their membranes and secrete them into the subendothelial space. Parthasarathy (14) reported that the ability of the artery wall cells to oxidize LDL is directly related to their ability to "seed" the LDL with reactive oxygen species. Navab and colleagues (17, 18) demonstrated in vitro, using human artery wall cell cocultures, that these cells were capable of creating microenvironments into which their oxidative waste could be secreted. At the same time, these microenvironments excluded aqueous antioxidants and allowed trapped LDL to undergo mild oxidation.

WHAT CAUSES MONOCYTES TO ENTER THE ARTERY WALL IN THE FIRST PLACE?

Napoli and colleagues (19) demonstrated that LDL accumulation and oxidation precede monocyte entry into the artery wall). Studying the arteries of human fetuses, they found that LDL was present and was oxidized before the entry of monocytes and that monocyte entry occurred at sites of oxidized LDL accumulation, strongly suggesting that LDL oxidation is a cause of monocyte entry and not just a by-product of monocyte metabolism (19). Berliner and colleagues (20–26) reported that mildly oxidized LDL, which was still recognized by the LDL receptor, stimulated human aortic endothelial cells to bind monocytes but not neutrophils and caused artery wall cells to produce the potent monocyte chemoattractant monocyte chemoattractant protein-1 and the differentiation factor monocyte colony stimulating factory. Subsequently, it was found that the biological activity of this minimally modified LDL (MM-LDL) was attributable to the oxidation of LDL phospholipids, which contain arachidonic acid in the sn-2 position (27–34). Direct measurement of these oxidized phospholipids in atherosclerotic lesions in animals suggested that they exist at concentrations that would be biologically active in vivo (29). Additionally, these oxidized phospholipids are generated in the membranes of cells that are under oxidative stress such as that caused by interleukin-1β (30) and are incorporated into apoptotic vesicles and blebs (35, 36).

THE OXIDIZED PHOSPHOLIPIDS IN MM-LDL ARE RECOGNIZED BY AUTOANTIBODIES

Palinski et al. (10) and Napoli et al. (19) demonstrated that an IgM monoclonal antibody isolated from apoE null

mice (EO6) *1*) binds to epitopes in human and animal atherosclerotic lesions, *2*) binds to epitopes necessary for macrophage recognition of oxidized LDL (37), and *3*) binds to apoptotic cells (38) and also recognizes the oxidized phospholipids in MM-LDL (37). The epitope recognized by the EO6 antibody was found to be structurally and functionally identical to classic "natural" T15 antiphospholipid antibodies that are of B-1 cell origin and are reported to provide protection from virulent pneumococcal infection (39). Subsequently, it was shown that vaccination against *Streptococcus pneumoniae* protected LDL receptor null mice from atherosclerosis (40). Thus, the oxidized phospholipids found in MM-LDL and the antibodies that naturally occur to these oxidized phospholipids appear to be part of the innate immune system (41, 42). Because sepsis is a major cause of fetal wastage and neonatal mortality, the presence of LDL-derived oxidized phospholipids that cause monocyte entry into the artery wall of the developing fetus and infant and the antibodies against these proinflammatory oxidized phospholipids, which also recognize bacteria, may provide a survival advantage. Indeed, Napoli and colleagues (19) found that maternal hypercholesterolemia enhanced fatty streak formation in fetal aortas. If, in fact, a survival advantage is conferred by the presence of the LDL-derived proinflammatory oxidized phospholipids, the antibodies against these oxidized lipids, and the monocytes that are induced to migrate as a result of the presence of these oxidized lipids, and this survival advantage is enhanced by maternal hypercholesterolemia, there would be positive evolutionary pressure for hypercholesterolemia. This might explain why hypercholesterolemia is so prevalent in human populations.

WHAT ARE THE MECHANISMS THAT CAUSE THE FORMATION OF THE OXIDIZED PHOSPHOLIPIDS FOUND IN MM-LDL?

A role for cell-generated reactive oxygen species in mediating LDL oxidation was postulated for many years (12–16). The first direct proof in vivo of a role for the lipoxygenase pathway was provided by Cyrus and colleagues (43–45) in apoE null mice that were made genetically deficient in 12/15-lipoxygenase and that were found to have significantly less atherosclerosis as a result. Deletion of the 12/15-lipoxygenase gene in LDL receptor null mice (46) and in macrophages of mice that were both LDL receptor null and also deficient in the apoB-editing catalytic polypeptide-1 enzyme (47) resulted in decreased atherogenesis in both mouse models. Conversely, overexpression of the 12/15-lipoxygenase gene in the endothelium of LDL receptor null mice accelerated atherosclerosis (48). Moreover, Hedrick and colleagues (49) generated transgenic mice in a C57BL/6J background that modestly overexpressed the murine 12/15-lipoxygenase gene. These mice had 2.5-fold increases in the levels of 12(*S*)-hydroxyeicosatetraenoic acid and a 2-fold increase in the expression of 12/15-lipoxygenase protein in vivo. These mice de-

veloped spontaneous aortic fatty streak lesions on a chow diet, further indicating the importance of the lipoxygenase pathway in atherogenesis. Navab and colleagues (50) found that human artery wall cells required the 12/15-lipoxygenase gene to generate the oxidized phospholipids found in MM-LDL.

Myeloperoxidase null mice had increased, not decreased, atherosclerosis (51), but mouse macrophages, unlike human macrophages, have little myeloperoxidase.

Myeloperoxidase reaction products have been found in human atherosclerotic lesions (52), and there is increasing evidence that the myeloperoxidase pathway can generate proinflammatory oxidized lipids (32, 53–58). Another pathway that may be a potential source of reactive oxygen species for the generation of proinflammatory oxidized lipids is the NADPH oxidase pathway (59, 60). **Figure 1** depicts the formation of the fatty acid hydroperoxides 13-hydroperoxyoctadecadienoic acid (HPODE) and
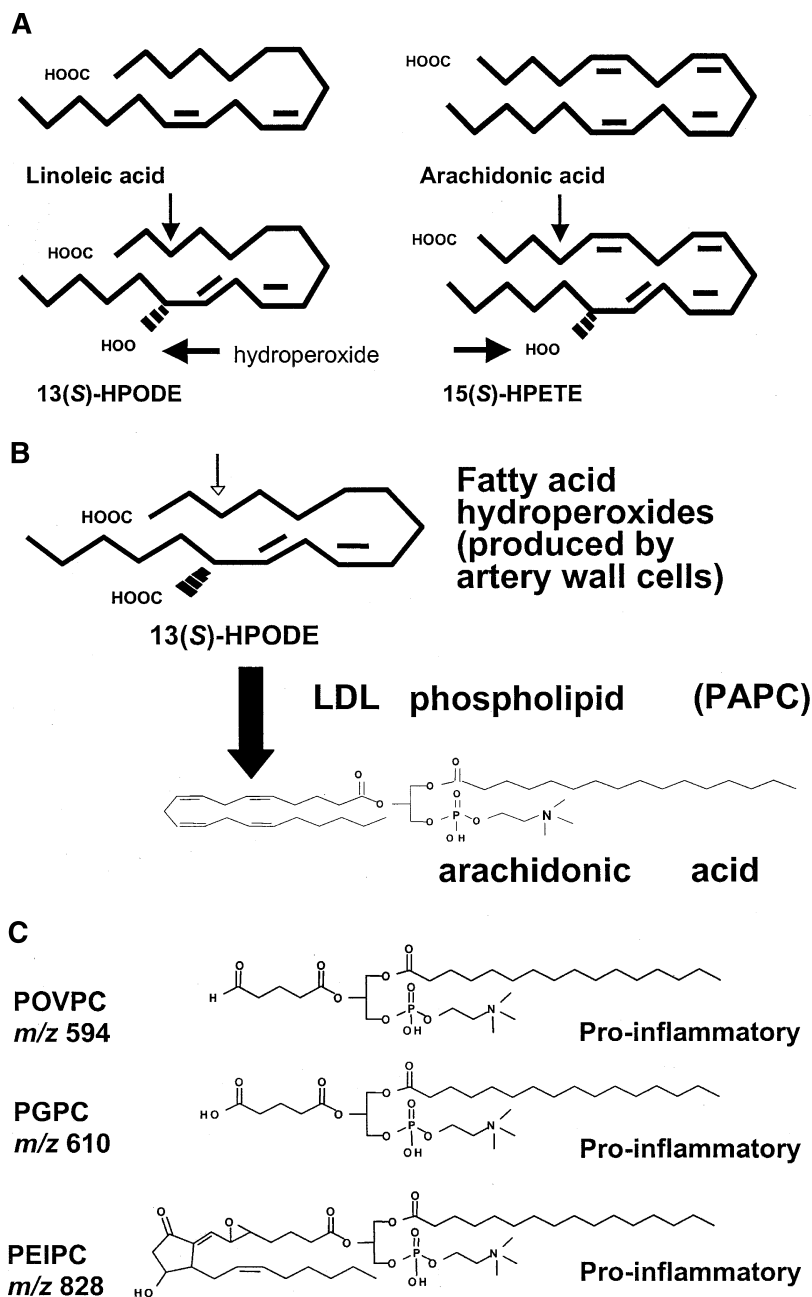

Fig. 1. A: The formation of the fatty acid hydroperoxides 13-hydroperoxyoctadecadienoic acid (HPODE) and 15-hydroperoxyeicosatetraenoic acid (HPETE). B: The action of HPODE on an LDL-derived phospholipid, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC). C: Three of the proinflammatory oxidized phospholipids in minimally modified LDL. POVPC, 1-palmitoyl-2-oxovaleryl-sn-glycero-3-phosphorylcholine; PGPC, 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphorylcholine; PEIPC, 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine.

15-hydroperoxyeicosatetraenoic acid (HPETE) (A), the action of HPODE on an LDL-derived phospholipid, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) (B), and the formation of three of the oxidized phospholipids in MM-LDL (C).

CHALLENGES TO THE OXIDATION HYPOTHESIS OF ATHEROGENESIS

The oxidation hypothesis of atherogenesis includes a large number of oxidized lipids. As indicated in the title of this paper, this review is focused on oxidized phospholipids and HDL. For a more broadly focused perspective, the reader is referred to a number of excellent recent reviews (32, 33, 42, 57, 61, 62).

Most of the in vitro studies on LDL oxidation were carried out with media containing relatively high levels of free metal ions. Heinecke appropriately questioned the applicability of these in vitro studies conducted in media rich in free metal ions to the situation in vivo (63). As shown in the experiments depicted in **Fig. 2**, a medium rich in free metal ions is not required for LDL oxidation or for the induction of monocyte chemotactic activity in the artery wall coculture system described by Navab et al. (18).
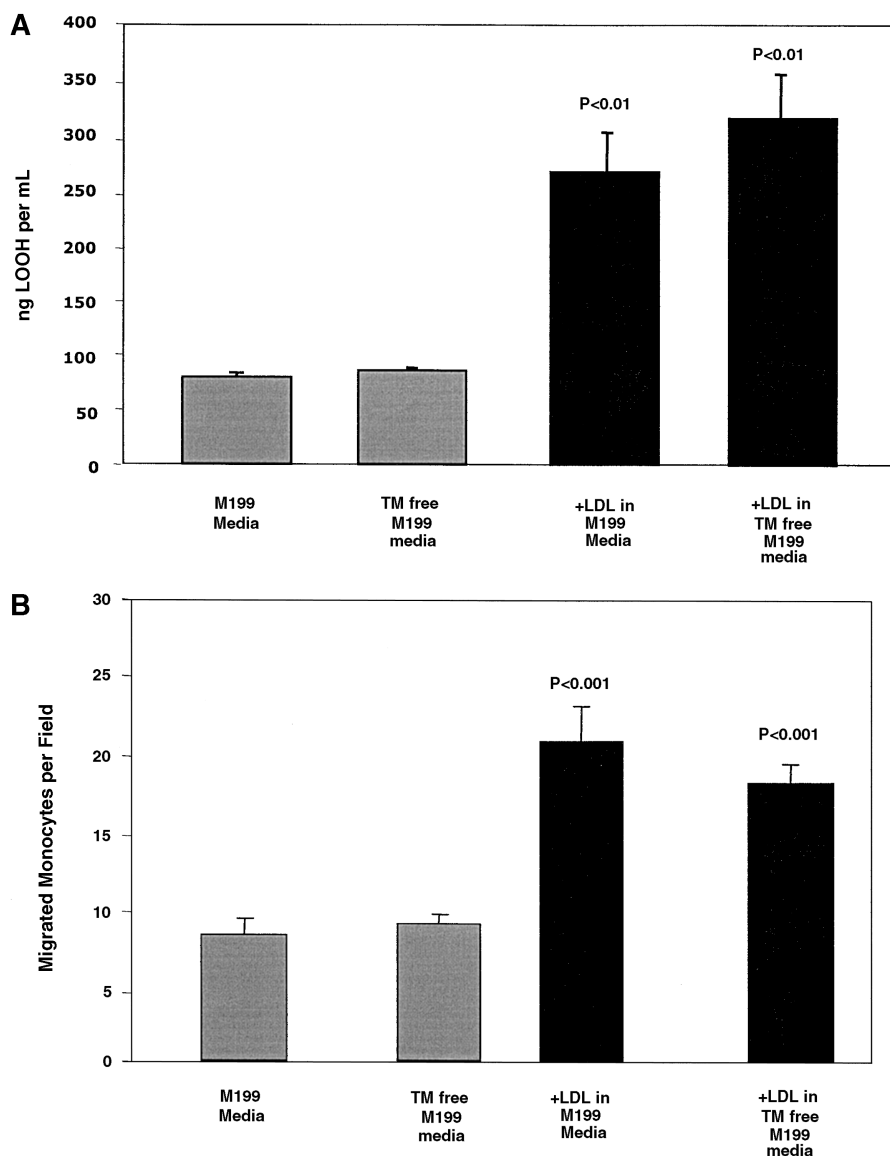

Fig. 2. Media rich in free metal ions are not required for LDL oxidation or the induction of monocyte chemotactic activity in cocultures of human artery wall cells. Artery wall cocultures were prepared as described (17) and maintained in either M199 medium or transition metal (TM)-free M199 medium (Fisher) without or with native human LDL (250 μg/ml) for 8 h. The supernatant was collected, and lipid hydroperoxide (LOOH) levels were determined by the Auerbach method (119) (A) or monocyte chemotactic activity was determined as described previously (82) (B). Error bars are SD.

Heinecke (63) and Stocker and colleagues (64) also questioned the oxidation hypothesis based on the findings of Chan and colleagues (65) that rabbits genetically engineered to specifically overexpress 15-lipoxygenase in their macrophages actually had less atherosclerosis. Additionally, Stocker and colleagues (64) treated Watanabe Heritable Hyperlipidemic (WHHL) rabbits with antioxidants (probucol and its metabolite bisphenol), resulting in decreased aortic cholesteryl ester and triglyceride peroxides without a decrease in aortic atherosclerosis in the bisphenol-treated animals but a statistically significant 31% decrease in en face lesions in the probucol-treated group.

The findings of Chan and colleagues (65) need to be considered in the context of studies by Zhao et al. (47) demonstrating that deficiency of the 12/15-lipoxygenase pathway in macrophages of mice that were LDL receptor null and also deficient in the apoB-editing catalytic polypeptide-1 enzyme resulted in significantly less atherosclerosis. Additionally, Harats et al. (48) and Reilly et al. (49) demonstrated that overexpression of 15-lipoxygenase in the endothelium of mice increased atherosclerosis. Conversely, Cyrus et al. (43, 45) demonstrated that disruption of the 12/15-lipoxygenase gene decreased atherosclerosis in apoE null mice, and George et al. (46) showed similar results in LDL receptor null mice. Moreover, specific pharmacological inhibition of 15-lipoxygenase in rabbits significantly reduced atherosclerosis (66, 67). Thus, on balance, the report by Chan and colleagues (65) is not strong evidence against the hypothesis.

With regard to the findings of Stocker and colleagues (64) that antioxidants decreased lesion cholesteryl ester and triglyceride peroxides without decreasing the cellular and other extracellular components of atherosclerosis in WHHL rabbits, it must be noted that these investigators did not measure, directly or by immunological means, the aortic content of the specific proinflammatory oxidized phospholipids (e.g., those recognized by the EO6 antibody). The data of Stocker and colleagues (64) would only speak against the oxidation hypothesis if they had shown that these specific proinflammatory oxidized phospholipids had been reduced without a reduction in aortic atherosclerosis, and they did not provide such evidence.

Similarly, the failure of vitamin E and other oral antioxidants to reduce measures of atherosclerosis in human clinical trials (68–70) must also be considered in this light. None of these trials provided evidence that the levels of the specific proinflammatory oxidized phospholipids were decreased by the oral antioxidants tested (71, 72). Indeed, these trials did not even show that administration of the antioxidants reduced lipid oxidation in the test subjects (71, 72). Moreover, FitzGerald and colleagues (73) demonstrated that doses of vitamin E as high as 2,000 IU/day for 8 weeks in human volunteers failed to reduce the excretion of specific isoprostanes that are a marker of oxidized phospholipids.

Heinecke (61) has indicated that vitamin E does not prevent lipid oxidation mediated by myeloperoxidase in vitro. Indeed, myeloperoxidase might actually convert an antioxidant to a pro-oxidant reaction (62). Navab et al. (18) reported that vitamin E could not inhibit the oxidation of LDL or prevent LDL-induced monocyte chemotactic activity in a coculture of human artery wall cells. As shown in **Fig. 3**, vitamin E also does not inhibit the lipoxygenase product 13(S)-HPODE from oxidizing the LDL-derived arachidonic acid-containing phospholipid PAPC. Thus, vitamin E is not able to prevent the formation of the specific proinflammatory oxidized phospholipids (74). Navab et al. (50) reported that 13(S)-HPODE was greater than 200 times more potent in oxidizing PAPC and forming these specific proinflammatory oxidized phospholipids than was hydrogen peroxide.

An example of the danger of extrapolating clinical trial data comes from a review of the history of the acceptance of thrombosis as an important component of the clinical events associated with atherosclerosis. Before 1950, acute myocardial infarction was assumed to be attributable to coronary thrombosis. During the 1950s, large-scale clinical trials used warfarin to prevent heart attack. The results were at best confusing. In this milieu of scientific confusion, a prominent pathologist suggested that thrombosis in myocardial infarction was a postmortem artifact. As a result, in the 1960s and 1970s, acute myocardial infarction was thought to be caused by a supply-demand imbalance in the myocardium. It was thought that a chronic narrowing of the coronary arteries as a result of atherosclerosis limited blood flow and that when demand increased without a concomitant increase in flow, myocardial infarction resulted. It was not until the advent of coronary arteriography during acute myocardial infarction and the introduction of potent thrombolytic agents and direct angioplasty for acute myocardial infarction in the 1980s and 1990s that the field came to clearly see the primary role that thrombosis plays in acute myocardial infarction. We now know that warfarin is an inadequate agent for preventing arterial thrombosis, in which platelets play a predominant role. Concluding that lipid oxidation is not cen-
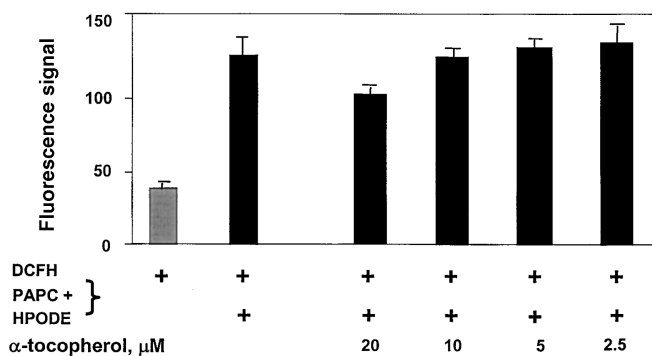

Fig. 3. Vitamin E cannot prevent the oxidation by 13(S)-HPODE of arachidonic acid in the LDL-derived phospholipid PAPC. Twenty micrograms of PAPC was incubated together with 1.0 μg of 13(S)-HPODE in the absence or presence of the concentration of vitamin E shown and in the presence of dichlorofluorescein (DCFH). Oxidation was determined by following the increase in fluorescence as previously described (74). The values are mean ± SD from two independent experiments.

tral to the development of atherosclerosis because of the failure of the antioxidants used to date in clinical trials could lead to an erroneous conclusion similar to the false belief that thrombosis was not important in the genesis of myocardial infarction because warfarin was ineffective in initial clinical trials.

Furthermore, genetic studies in humans and mice are consistent with a role of lipid oxidation in atherogenesis. Paraoxonase1 (PON1) can prevent the formation of and destroy the proinflammatory oxidized phospholipids in vitro, and PON1 null mice have increased atherosclerosis (75, 76), whereas PON1 transgenic mice have decreased atherosclerosis (77). Also, more than 20 epidemiologic studies have shown an association between polymorphisms of PON1 and heart disease in human populations (78). Additionally, studies modulating other antioxidant genes, such as heme oxygenase-1 (79–81), suggest that lipid oxidation is a primary cause of inflammation in atherosclerosis.

THE ROLE OF HDL IN ATHEROGENESIS

In contrast to vitamin E, HDL, apoA-I, and apoA-I mimetic peptides have been shown to prevent LDL oxidation in cell-free systems (1, 13, 14) and in the artery wall coculture studies of Navab et al. (50, 82). Moreover, HDL, apoA-I, and apoA-I mimetics have been shown to decrease lesions and improve vascular reactivity in animal models of atherosclerosis (83–90) and in humans (91–93).

The primary mechanism by which HDL and apoA-I and apoA-I mimetic peptides exert their beneficial effect has been presumed to be the enhancement of reverse cholesterol transport (85, 94–96). However, apoA-I has also been shown to be capable of removing "seeding molecules"

from LDL, thus preventing the oxidation of LDL-derived phospholipids to those that are thought to be responsible for the inflammatory response characteristic of atherosclerosis (50, 82).

Sevanian and colleagues (97) noted that a subpopulation of freshly isolated LDL contains lipid hydroperoxides. Navab et al. (82) found that freshly isolated LDL from normal individuals always contained small amounts of lipoxygenase pathway products (e.g., HPODE and HPETE). These were present even when blood was collected into tubes containing potent antioxidants. The levels of HPODE and HPETE did not increase during in vitro incubations in the presence of these antioxidants, indicating that they were present in LDL in vivo. However, when the freshly isolated LDL was incubated with apoA-I in the presence of antioxidants and the LDL and the apoA-I were then rapidly separated, the LDL treated with apoA-I contained only approximately one-third to one-half as much HPODE and HPETE as was present initially. Before these incubations, the apoA-I contained no detectable HPODE or HPETE, but after the incubation with LDL, one-half to two-thirds of the HPODE and HPETE that had been present in the LDL was transferred to the apoA-I, along with some cholesterol and phospholipid. The LDL treated with apoA-I was unable to generate lipid hydroperoxides, nor was it able to induce monocyte adherence or monocyte chemotactic activity when added to human artery wall cocultures. If the apoA-I that was incubated with the LDL was subjected to lipid extraction and the extracted lipids were added back to the LDL that had been treated with apoA-I, the reconstituted LDL was able to induce lipid hydroperoxide formation and induce monocyte adherence and monocyte chemotactic activity (82). Consistent with these properties of apoA-I, Bowry, Stanley, and Stocker (98) reported that HDL is a major
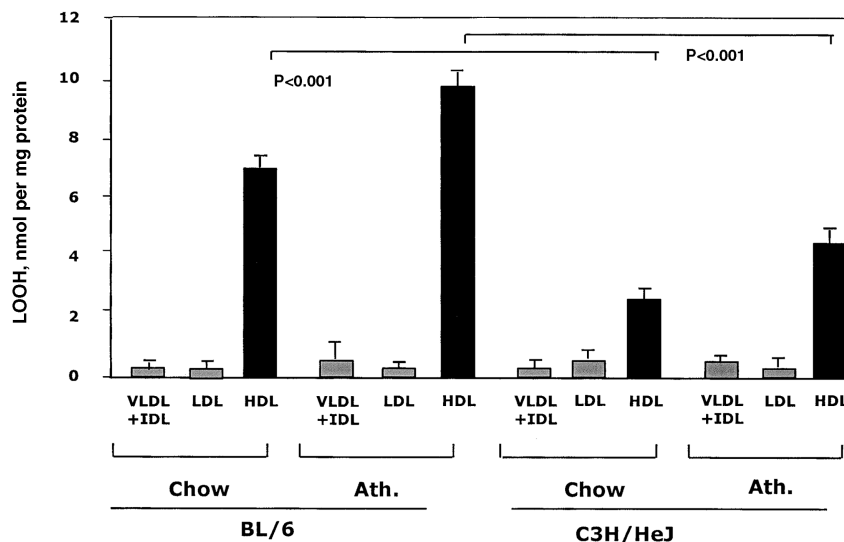

Fig. 4. LOOH content in C57BL/6J (BL/6) and C3H/HeJ mouse lipoproteins. The mice were maintained on a low-fat chow diet or on an atherogenic (Ath.) diet (106), their lipoproteins were separated by fast-protein liquid chromatography (FPLC) (119), and the LOOH content was determined as described (119, 120). IDL, intermediate density lipoprotein. The values are mean ± SD from two independent experiments.

carrier of lipid hydroperoxides in humans. As shown in **Fig. 4**, HDL appears to be the major carrier of lipid hydroperoxides in mice, and the concentration of lipid hydroperoxides in HDL taken from the atherosclerosis-susceptible C57BL/6J mice either on a low-fat chow diet or on an atherogenic diet was significantly greater than the lipid hydroperoxide levels found in the HDL of the atherosclerosis-resistant C3H/HeJ mice.

Six hours after injection of human apoA-I (100 μg/mouse) into the tail veins of C57BL/6J mice, their LDL was no longer able to induce lipid hydroperoxide formation or monocyte chemotactic activity in human artery wall cocultures (82). In contrast, injection of human apoA-II (100 μg/mouse) or saline did not prevent LDL-mediated induction of lipid hydroperoxide formation or LDL-induced monocyte chemotactic activity. Similarly, 6 h after infusing apoA-I and phospholipid into healthy human volunteers, there was a dramatic decrease in the ability of their LDL to induce lipid hydroperoxide formation and monocyte chemotactic activity in the cocultures of six of six subjects. Thus, apoA-I has the ability to remove HPODE and HPETE from human LDL and to dramatically reduce the inflammatory properties of LDL in both mice and humans (82).

HDL INFLAMMATORY INDEX

The ability of apoA-I to promote reverse cholesterol transport and remove the seeding molecules from LDL that are necessary for the formation of the inflammatory LDL-derived oxidized phospholipids likely contributes to the inverse relationship between HDL-cholesterol levels and susceptibility to atherosclerosis. Although the inverse relationship between HDL-cholesterol levels and risk of acute myocardial infarction is highly significant, the relationship is far from perfect. In the Air Force/Texas Coronary Atherosclerosis Prevention Study, subjects with "average" total cholesterol levels were followed for an average of 5.2 years. Of those given placebo, the event rate during the study was 2.1, 2.9, and 3.4% for those with HDL-cholesterol levels of ≥ 40, 35–39, and ≤ 34 mg/dl, respectively. Although the differences were highly significant, knowledge of the HDL-cholesterol level in predicting whether a specific individual would or would not have an event was clearly of limited use (99, 100).

Similarly, in the original Framingham Study, the incidence of coronary heart disease (CHD) was compared with HDL-cholesterol levels (101). With minimal assumptions, one can calculate from the published data that 44% of the events occurred in men with HDL-cholesterol levels of ≥ 40 mg/dl and 43% of the events occurred in women with HDL-cholesterol levels of ≥ 50 mg/dl (100).

Because a significant number of CHD events occur in patients with normal LDL-cholesterol levels and normal HDL-cholesterol levels (100–102), there has been a continuing search for markers with better predictive value (100, 103).

One of the first indications that HDL might be such a marker came from the studies of Van Lenten et al. (104). They reported that the acute phase response in humans converted HDL from anti-inflammatory to proinflammatory. These studies compared HDL taken from humans before and after elective surgery. Before surgery, HDL was anti-inflammatory in a human artery wall cell coculture model (i.e., HDL inhibited LDL oxidation and LDL-induced monocyte chemotactic activity). However, at the peak of the acute phase response, 3 days after surgery, HDL from the same patient was proinflammatory (i.e., it promoted LDL oxidation and monocyte chemotactic activity in the human artery wall coculture). One week after surgery, the HDL returned to an anti-inflammatory state (100, 104). These changes in HDL are consistent with a classic acute phase response. Gabay and Kushner (105) emphasized that the acute phase response can become chronic. Navab et al. (106) demonstrated that such a chronic acute phase response was present in apoE null mice on a chow diet and in LDL receptor null mice on a high-fat diet and was accompanied by proinflammatory HDL.

In addition to apolipoproteins such as apoA-I, HDL also contains enzymes that can prevent the formation of or destroy the oxidized phospholipids that mediate the inflammatory response induced by MM-LDL (107). These enzymes include paraoxonase (75–77), platelet-activating factor acetylhydrolase (108), lecithin:cholesterol acyltransferase (109), and possibly glutathione peroxidase (107). In vivo, the absence of the HDL-associated enzyme paraoxonase was demonstrated to result in increased LDL oxidation and increased atherosclerosis (76). Van Lenten et al. (104) demonstrated that the acute phase response in rabbits and humans resulted in decreased activities of HDL-associated paraoxonase and platelet-activating factor acetylhydrolase.

An example of a chronic acute phase response in humans is the persistent increase (highest tertile) of the positive acute phase reactant C reactive protein (CRP) in the absence of a detectable infection or other acute stress (103).

Navab et al. (74) reported that the inflammatory/anti-inflammatory properties of HDL from 27 normolipidemic CHD patients clearly separated the patients from 31 age- and gender-matched controls. Each of the patients had at least a 50% narrowing of a coronary artery, none smoked, none was diabetic, none was taking hypolipidemic medications, and all had normal blood lipids, as defined by total cholesterol of <200 mg/dl, LDL-cholesterol of <130 mg/dl, triglycerides of <150 mg/dl, and HDL-cholesterol of >40 mg/dl for males and >50 mg/dl for females. The patient HDL, in contrast to control HDL, was proinflammatory in the human artery wall cell coculture model and in a cell-free assay. These patients had no evidence of an acute illness that could explain an acute phase response. Thus, Navab et al. (74) postulated that the inflammatory properties of HDL in these patients represented a "chronic" acute phase response similar to that described by CRP levels in the top tertile of "normal" (103).

Ansell et al. (100) studied two additional patient groups. Group I comprised 26 patients who presented

with stable CHD or CHD equivalents by National Cholesterol Education Program Adult Treatment Panel-III criteria (110) who were not yet on a statin or another hypolipidemic agent and whose physician recommended treatment with a statin. The inflammatory/anti-inflammatory properties of HDL from these patients were compared before and 6 weeks after starting statin therapy. A second group of patients were studied who presented with high HDL-cholesterol levels and documented CHD (100). The inflammatory/anti-inflammatory properties of the HDL from both groups of patients were compared with those of age- and gender-matched healthy controls using an HDL inflammatory index. The HDL inflammatory index was measured in two ways. The first was a cell-based assay in which the monocyte chemotactic activity generated in a human artery wall cell coculture by a standard control LDL was determined in the absence and presence of the test HDL. The monocyte chemotactic activity determined in the absence of the test HDL was normalized to 1.0. The monocyte chemotactic activity obtained when the test HDL was added was then compared with the monocyte chemotactic activity in the absence of HDL. If the value after adding the test HDL was greater than that obtained without the test HDL (i.e., HDL inflammatory index > 1.0), the test HDL was classified as proinflammatory. On the other hand, if the value obtained after addition of the test HDL was less than that without the test HDL (i.e., HDL inflammatory index < 1.0), the HDL was classified as anti-inflammatory.

Ansell et al. (100) also used a cell-free assay in which 1-palmitoyl-2-(5,6-epoxyisoprostane E(2))-sn-glycero-3-phosphorylcholine (PEIPC) (Fig. 1C) was added to a cell-free system containing dichlorofluorescein (DCFH) (similar to the system described in Fig. 3). PEIPC was chosen because this oxidized phospholipid accounts for more than 80% of the LDL-induced monocyte chemotactic activity in human artery wall cell cocultures (i.e., 80% of the monocyte chemotactic activity resulting from the addition of LDL to the cocultures is attributable to the formation of PEIPC). The fluorescent signal generated by PEIPC in the absence of the test HDL was normalized to 1.0. If the signal increased after addition of the test HDL (i.e., >1.0), the test HDL was classified as proinflammatory. If the fluorescent signal decreased after addition of the test HDL (i.e., <1.0), then the HDL was classified as anti-inflammatory.

As shown in **Fig. 5A**, the patients in Ansell's group I had proinflammatory HDL before statin therapy. After 6 weeks of simvastatin (40 mg/day) (100), their HDL was less proinflammatory but was still proinflammatory. In contrast, the healthy age- and gender-matched controls had anti-inflammatory HDL. Figure 5B demonstrates similar results for the cell-free assay. **Figure 6** shows the HDL lipid hydroperoxide content for Ansell's group I patients and controls. The lipid hydroperoxide content of the patients' HDL was significantly greater than that of the healthy controls (Fig. 6). After 6 weeks of simvastatin therapy, there was a trend toward lower levels of HDL lipid hydroperoxides, but this did not reach significance ($P = 0.07$) (100).
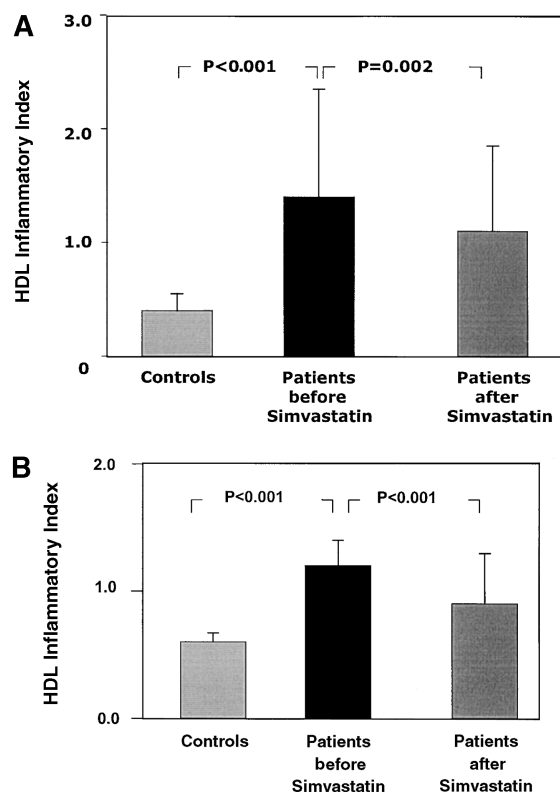
Fig. 5. A: The HDL inflammatory index was determined in human artery wall cell cocultures for group I from Ansell et al. (100). The data shown are for patients before and after simvastatin treatment (40 mg/day for 6 weeks) and for healthy age- and gender-matched controls. B: The HDL inflammatory index was determined in a cell-free assay for group I from the same study. The data shown are for patients before and after simvastatin treatment (40 mg/day for 6 weeks) and for healthy age- and gender-matched controls. Values shown are means ± SD.

Figure 7 demonstrates that the HDL from the group II patients, 20 patients with HDL-cholesterol levels ranging between 84 and 148 mg/dl, none of whom was taking a statin or other hypolipidemic medication and all of whom had documented CHD [group II in ref. (100)], was proinflammatory.

In Ansell's study (100), the HDL inflammatory index separated patients from the healthy controls much better than did HDL-cholesterol levels. In group I, only 3 of 26 patients had abnormally low HDL-cholesterol levels before simvastatin therapy, whereas 20 of 26 patients had an HDL inflammatory index of ≥1.0 and all 26 patients had an HDL inflammatory index of >0.6, compared with all 26 of the controls, whose values were <1.0 by the coculture assay. Twenty-four of the 26 controls had an HDL inflammatory index of <0.6 by the coculture assay. After 6 weeks of simvastatin therapy, there was a highly significant reduction in the inflammatory properties of the HDL in group I patients, but their HDL remained significantly more inflammatory than did the HDL from controls.

In patient group II, which was composed of patients referred for study because of CHD and high HDL-cholesterol levels (95 ± 14 mg/dl), only one patient had an in-
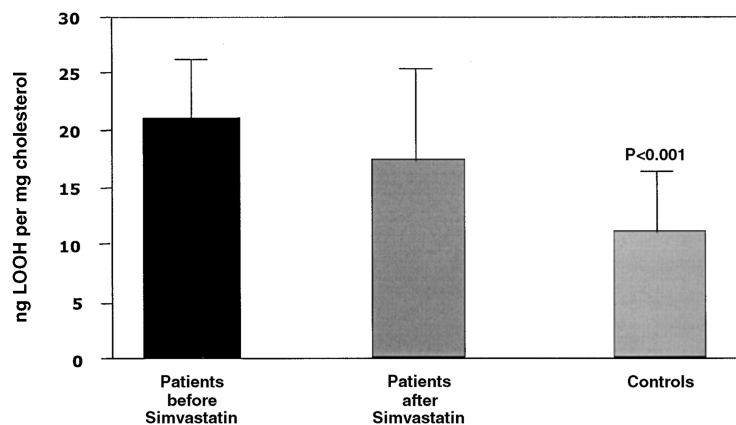
Fig. 6. HDL-LOOH content was determined for group I from Ansell et al. (100). The data shown are for patients before and after simvastatin treatment (40 mg/day for 6 weeks) and for healthy age- and gender-matched controls. Values shown are means ± SD.

creased LDL-cholesterol level (>160 mg/dl), only two patients had increased triglycerides (>150 mg/dl), and none was diabetic (100). None of these 20 patients was on a statin or other hypolipidemic agent. Eighteen of these 20 patients had an HDL inflammatory index of ⩾1.0, and only one had an HDL inflammatory index of <0.6, whereas all 20 of the controls had an HDL inflammatory index of <0.6 by the coculture assay.

Because there were significant correlations between HDL-lipid hydroperoxides and the HDL inflammatory index determined in either the coculture or the cell-free assay (100), one might ask if the HDL inflammatory index is simply a measure of HDL-lipid hydroperoxide. If this were the case, adding normal HDL would always give a value of >1.0 in the cell-free assay, because all of the controls had some lipid hydroperoxide in their HDL (100). However, of the 46 healthy control subjects studied by Ansell and colleagues (26 for group I and 20 for group II), only one had an HDL inflammatory index of >1.0 as determined by the cell-free assay. Navab et al. (107) reported that the

HDL inflammatory index measures the net action of a large number of factors in HDL. These factors include oxidized phospholipids, lipid hydroperoxides, paraoxonase activity, platelet-activating factor acetylhydrolase activity, lecithin:cholesterol acyl transferase activity, possibly GSH peroxidase activity, apoA-I, apoJ, serum amyloid A, ceruloplasmin, antioxidant vitamins, and probably products such as nitrotyrosine, which can be generated by myeloperoxidase. Thus, the HDL inflammatory index likely represents the net effect of all of these factors. The lipid hydroperoxide content and the other factors in HDL are likely interdependent.

The correlation of lipid hydroperoxide content with HDL that is less able to prevent the formation of the LDL-derived proinflammatory oxidized phospholipids (100) suggests that oxidation impairs HDL function. Macdonald et al. (111) reported that oxidation of HDL per se does not make HDL less functional. They reported that tyrosyl radical oxidation of mouse HDL induced the formation of apoA-I/A-II heterodimers (tyrHDL), which enhanced the
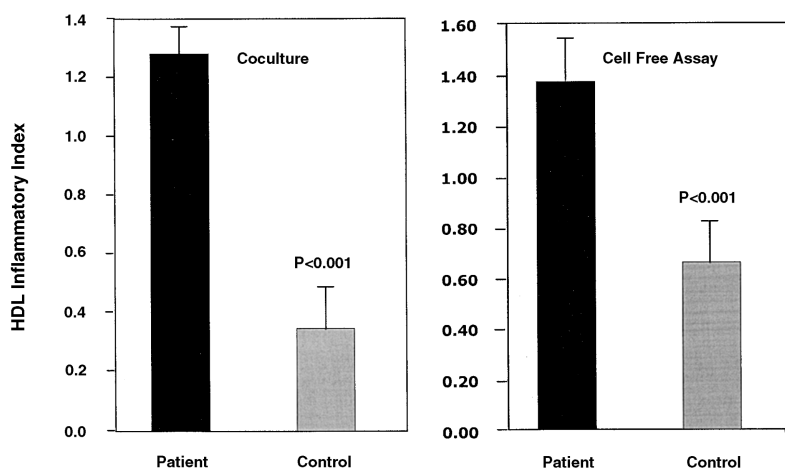

Fig. 7. The HDL inflammatory index was determined in human artery wall cell cocultures and in the cell-free assay for group II from Ansell et al. (100), which was composed of patients with high levels of HDL-cholesterol and documented coronary heart disease. The data shown are for the patients and age- and gender-matched healthy controls. Values shown are means ± SD.
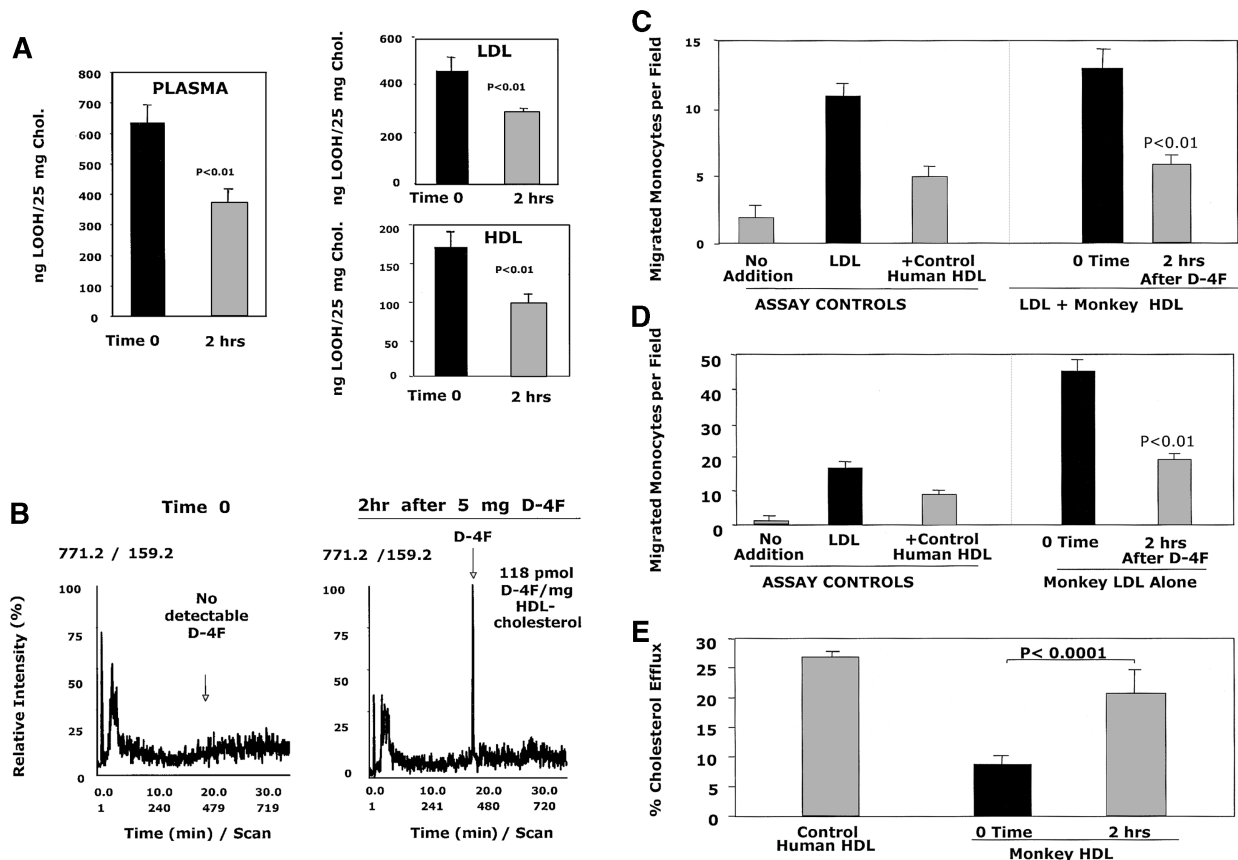
Fig. 8. An oral apolipoprotein A-I mimetic peptide, D-4F, reduces monkey lipoprotein LOOH, converts monkey HDL to anti-inflammatory, and increases monkey HDL-mediated cholesterol (Chol.) efflux from human monocyte macrophages. Three 1 year old nonnaïve, 3 kg female cynomolgus monkeys maintained on a Purina chow diet were given by stomach tube a single dose of 5.0, 50, or 500 mg of D-4F (87) in 25 ml of water. Blood samples were drawn before dosing (Time 0) and 2 h after D-4F administration. A: Plasma was isolated and fractionated by FPLC (120), and fractions containing HDL and LDL were assayed for LOOH as described (119, 120). B: HDL content of D-4F was determined by liquid chromatography multiple reaction monitoring (121) and normalized to HDL-cholesterol. C and D: The ability of the monkey HDL to prevent oxidized phospholipid (PAPC + HPODE)-mediated induction of monocyte chemotactic activity in a human artery wall coculture (74) was also determined (C), as was the ability of monkey LDL to induce monocyte chemotactic activity in the coculture (87) (D). E: The ability of the monkey HDL to promote cholesterol efflux from human monocyte macrophages was also determined and compared with that of normal (control) human HDL (120). The results shown are for the monkey that received 5 mg of oral D-4F; similar results were obtained with the higher doses (data not shown). Values shown are means ± SD.

ability of mouse HDL to deplete cultured fibroblasts of their regulatory pool of cholesterol. When apoE null mice were injected intraperitoneally twice weekly with 150 μg of tyrHDL from age 10 to 18 weeks, there was a maximum 2.3-fold increase in endogenous HDL-cholesterol levels, which decreased toward the end of the treatment period. Treatment with tyrHDL resulted in 37% less aortic lesion development than in control HDL-treated mice ($P <$ 0.001) and 67% less than in saline-injected animals ($P <$ 0.001). Macdonald et al. hypothesized that increasing the mobilization of cellular cholesterol to apoA-I could decrease atherosclerosis without necessarily causing sustained increases in circulating HDL-cholesterol levels.

In an accompanying editorial, Bergt, Oram, and Heinecke (112) suggested that cross-linked heterodimers of apoA-I and apoA-II in tyrosylated HDL may be responsible for its enhanced ability to remove cholesterol from lipid-laden cells (113). These authors (112) speculated that

heterodimers may act more like lipid-free apolipoproteins, perhaps because of conformational changes in apoA-I that expose more amphipathic α-helices to ABCA1. They also noted that apoA-I mimetic peptides synthesized from D-amino acids appear to retard atherogenesis in hypercholesterolemic mice without affecting HDL levels and that in vitro studies have suggested that this effect is mediated in part by inhibition of LDL oxidation (87). Bergt, Oram, and Heinecke (112) suggested the possibility that such apoA-I mimetic peptides (87) might also promote reverse cholesterol transport, but this had not been investigated. Additionally, they commented that HDL appears to be the major carrier of lipid hydroperoxides in the plasma and may transport oxidized cholesteryl esters to the liver for excretion (114). They concluded that HDL might also decrease the risk of atherosclerosis by altering the structures or metabolic fates of oxidized lipids that would otherwise exert atherogenic effects (112).

IS THERE A RELATIONSHIP BETWEEN THE INFLAMMATORY PROPERTIES OF HDL AND THE ABILITY OF HDL TO MEDIATE CELLULAR CHOLESTEROL EFFLUX?

Grunfeld and colleagues (115) reported that HDL taken from Syrian hamsters in which an acute phase reaction was induced by lipopolysaccharide injection was less able to mediate cellular cholesterol efflux than was HDL taken from control animals. Pussinen et al. (116) reported that HDL-mediated cholesterol efflux significantly improved in patients with periodontitis who were PCR positive for *Actinobacillus actinomycetemcomitan* and whose CRP decreased (53.7% reduction; $P = 0.015$) after treatment. They concluded that periodontitis causes similar, but milder, changes in HDL metabolism than those that occur during the acute phase response and that periodontitis may diminish the antiatherogenic potency of HDL.

Reddy and colleagues (117) reported that LDL oxidation by human artery wall cells was controlled by the cholesterol content of the cells, which was determined in part by ABCA1 activity. They proposed that the reverse cholesterol transport hypothesis of atherogenesis and the oxidation hypothesis of atherogenesis might be two sides of the same coin. The preliminary data shown in **Fig. 8** are consistent with the proposal by Reddy et al. (117). A single oral administration of D-4F, an oral apoA-I mimetic peptide, to cynomolgus monkeys at doses as low as 1.67 mg/kg reduced plasma and lipoprotein lipid hydroperoxides 2 h after the dose (Fig. 8A). Two hours after a single oral administration of D-4F at 1.67 mg/kg, the monkey HDL contained 118 pmol of D-4F per milligram of HDL-cholesterol (Fig. 8B). Concomitant with the decrease in lipoprotein lipid hydroperoxides, there was a significant improvement in the monkey HDL anti-inflammatory properties (Fig. 8C) and a decrease in the ability of the monkey LDL to induce human artery wall cells to produce monocyte chemotactic activity (Fig. 8D). Paralleling these changes was a dramatic increase in the ability of the monkey HDL to promote cholesterol efflux from human monocyte macrophages (Fig. 8E).

SUMMARY AND CONCLUSIONS

Scientific controversy on subjects such as the role of thrombosis in acute myocardial infarction and the oxidation hypothesis of atherogenesis may be taken in a broader human context. In speaking to the House of Commons on November 12, 1940, on the occasion of the passing of Neville Chamberlain, Winston S. Churchill said:

> At the lychgate we may all pass our own conduct and our own judgments under a searching review. It is not given to human beings, happily for them, for otherwise life would be intolerable, to foresee or to predict to any large extent the unfolding course of events. In one phase men seem to have been right, in another they seem to have been wrong. Then again, a few years later, when the perspective of time has lengthened, all stands in a different setting. There is a new proportion. There is another scale of values. History with its flickering lamp stumbles along the trail of the past, trying to reconstruct its scenes, to revive its echoes, and kindle with pale gleams the passion of former days. What is the worth of all this? The only guide to a man is his conscience; the only shield to his memory is the rectitude and sincerity of his actions. It is very imprudent to walk through life without this shield, because we are so often mocked by the failure of our hopes and the upsetting of our calculations; but with this shield, however the Fates may play, we march always in the ranks of honor. (118) (p. 550)

There is continuing evidence of a role for lipid oxidation in atherogenesis. The oxidation hypothesis of atherogenesis has evolved to focus on specific proinflammatory oxidized phospholipids that result from the oxidation of LDL phospholipids containing arachidonic acid and that are recognized by the innate immune system in animals and humans. These oxidized phospholipids are generated via the lipoxygenase and myeloperoxidase pathways. Among many possible reasons, the failure of antioxidant vitamins to influence clinical outcomes may result from the failure of vitamin E to prevent the formation of these proinflammatory oxidized phospholipids. Preliminary data suggest that the oxidation hypothesis of atherogenesis and the reverse cholesterol transport hypothesis of atherogenesis may be linked. Measurements of the HDL inflammatory index and of the levels of oxidized lipids in lipoproteins and products of the myeloperoxidase pathway may predict susceptibility to atherogenesis. ApoA-I and apoA-I mimetic peptides may reduce oxidized lipids and also improve reverse cholesterol transport and, therefore, may have therapeutic potential.

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