Vascular Lipid Accumulation, Lipoprotein Oxidation, and Macrophage Lipid Uptake in Hypercholesterolemic Zebrafish


Abstract—Lipid accumulation in arteries induces vascular inflammation and atherosclerosis, the major cause of heart attack and stroke in humans. Extreme hyperlipidemia induced in mice and rabbits enables modeling many aspects of human atherosclerosis, but microscopic examination of plaques is possible only postmortem. Here we report that feeding adult zebrafish (Danio rerio) a high-cholesterol diet (HCD) resulted in hypercholesterolemia, remarkable lipoprotein oxidation, and fatty streak formation in the arteries. Feeding an HCD supplemented with a fluorescent cholesteryl ester to optically transparent fli1:EGFP zebrafish larvae in which endothelial cells express green fluorescent protein (GFP), and using confocal microscopy enabled monitoring vascular lipid accumulation and the endothelial cell layer disorganization and thickening in a live animal. The HCD feeding also increased leakage of a fluorescent dextran from the blood vessels. Administering ezetimibe significantly diminished the HCD-induced endothelial cell layer thickening and improved its barrier function. Feeding HCD to hyz:DsRed2 larvae in which macrophages and granulocytes express DsRed resulted in the accumulation of fluorescent myeloid cells in the vascular wall. Using a fluorogenic substrate for phospholipase A2 (PLA2), we observed an increased vascular PLA2 activity in live HCD-fed larvae compared to control larvae. Furthermore, by transplanting genetically modified murine cells into HCD-fed larvae, we demonstrated that toll-like receptor-4 was required for efficient in vivo lipid uptake by macrophages. These results suggest that the novel zebrafish model is suitable for studying temporal characteristics of certain inflammatory processes of early atherogenesis and the in vivo function of vascular cells. (Circ Res. 2009;104:952-960.)

Key Words: zebrafish ■ atherosclerosis ■ oxidized lipoprotein ■ macrophage

Current experimental studies of atherosclerosis often use genetically modified mice fed high-fat, high-cholesterol diets, which rapidly induce extreme hyperlipidemia and lipid accumulation in the artery wall. One important limitation of using mice is the difficulty in studying the temporal course of pathogenic events because microscopic examination of atherosclerotic lesions can be performed only postmortem. In this regard, an advantage of using zebrafish (Danio rerio) is that their larvae are optically transparent until about the 30th day of development, which enables temporal observations of fluorescent proteins and probes in a live animal. Transgenic fli1:EGFP zebrafish, which express enhanced green fluorescent protein (GFP) in the vascular endothelium, have been imaged extensively in high resolution using confocal microscopy to analyze developmental angiogenesis and tumor cell intravasation in live animals.1,2 Thus, if one could induce hyperlipidemia and lipid accumulation in blood vessels in fli1:EGFP zebrafish, this will create a valuable model for in vivo monitoring of early pathological processes of atherogenesis.

Fish are poikilothermic vertebrates that preferentially use lipids rather than carbohydrates as an energy source and would be classified, using standards applied to mammals, as mildly hyperlipidemic and hypercholesterolemic.3 In 1962, Vastesaeger and Delcourt observed the presence of lipid-rich atherosclerosis-like lesions in the aorta of a tuna (Thunnus thynnus).4 More recently, Seierstad et al demonstrated similar lesions in coronary arteries of farmed Atlantic salmon (Salmo salar).5 Lipoproteins have been studied in teleost fish, particularly in rainbow trout (Oncorhynchus mykiss).3 Very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) classes have been identified by analytic ultracentrifugation, with HDL dominating the lipoprotein profile. The nature and the distribution of

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In this study, we sought to explore the potential of zebrafish for atherosclerosis-related research. Zebrafish (Danio rerio) are a popular model organism for studying human diseases because they share many genetic similarities with humans. This similarity allows for the direct application of findings from zebrafish studies to human medicine.

### Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at [http://circres.ahajournals.org](http://circres.ahajournals.org).

### Zebrafish

Zebrafish were obtained from the Zebrafish International Resource Center (ZFIN) and maintained in a temperature-controlled facility. Fish were fed a regular diet of commercial fish food and provided fresh water daily. Zebrafish were housed in a temperature-controlled facility at 28°C with a 14:10 light:dark cycle.

### Lipid and Lipoprotein Analyses

Blood samples were collected from the caudal vein of adult zebrafish using heparinized microcapillaries. Plasma was isolated by centrifugation at 4°C and stored at -80°C until analysis. Lipid and lipoprotein levels were determined using automated enzymatic assays (Roche Diagnostics and Equal Diagnostics). Plasma lipoprotein profiles were analyzed by agarose gel electrophoresis (Helena Laboratories). Oxidation-specific epitopes were detected in an immunoblot assay with E06 monoclonal antibody.

### Histology

Tissue specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for histological analysis. Sections were stained with hematoxylin and eosin (H&E) or with antibodies specific to various cellular markers.

### In Vivo Microscopy

For in vivo confocal microscopy, anesthetized fish larvae were housed in a sealed, temperature-controlled chamber in a small drop of tricaine containing water. A Nikon C1si confocal microscope was used to observe the vascular lipid accumulation experiments described in the online data supplement.

### Lipoprotein Oxidation in Adult Zebrafish

To test whether zebrafish are inherently susceptible to high-cholesterol feeding, HCD was fed to zebrafish starting at 5 weeks postfertilization (“adult” fish) for an additional 8 to 12 weeks. Compared to control animals who received normal food, HCD-fed zebrafish had an enlarged belly (Figure 1A), but the weight gain was not statistically different (Figure 1B). However, there was a dramatic, 4-fold increase in plasma TC levels, reaching on average 800 mg/dL (Figure 1C), values observed in cholesterol-fed LDLR−/− mice developing atherosclerosis. Elevated TC levels in HCD-fed fish were found as early as at 40 to 45 days post fertilization (dpf) (Figure 1 in the online data supplement) and likely develop even earlier, but we were unable to collect blood from younger fish. The triglyceride levels were not statistically different (Figure 1D) possibly because no fat was added to the HCD. Agarose native gel electrophoresis followed by Fat Red staining demonstrated that control zebrafish plasma contained a distinct lipoprotein fraction corresponding to human HDL as well as other unresolved bands (Figure 1E). This agrees with the reports of HDL dominating the lipoprotein profile in other teleost fish. In contrast, plasma from the HCD-fed zebrafish had, in addition to a prominent HDL fraction, strong bands that appear to correspond to human LDL and VLDL. Interestingly, many plasma samples from HCD-fed fish contained high-mobility bands (we show 3 representative samples in Figure 1E), which may correspond to electronegative, oxidized LDL.

### Lipoprotein Oxidation in Adult Zebrafish

Our present understanding of atherogenesis considers that the oxidative modification of LDL is a leading factor in the development of atherosclerosis. The results presented here support this hypothesis, as we observed significant increases in plasma TC levels and the development of atherosclerotic lesions in HCD-fed zebrafish. The use of zebrafish as a model organism for studying human diseases is a promising strategy that can lead to new insights into the mechanisms of atherosclerosis and potential therapeutic targets.
initiation and progression of the atherosclerotic lesion. Our laboratory has developed monoclonal antibodies that can be used in immunoassays to detect oxidation-specific epitopes on lipoproteins in plasma of different animal species and humans. Monoclonal antibody EO6 is used to measure the amount of oxidized phospholipids bound per apoB or apoAI lipoproteins. In human epidemiological studies, a 3- to 5-fold increase in the EO6/apoB plasma levels multiplies the risk of coronary artery disease 3-fold among patients 60 years of age and when combined with hypercholesterolemia, up to 17-fold.

Thus, given high TC levels and evidence for elevated VLDL and LDL lipoproteins, we measured the EO6/apoB levels in zebrafish plasma. We found that a polyclonal antibody against human apoB recognized proteins in zebrafish plasma corresponding to human apoB (Online Figure II), which agrees with a similar cross-reactivity of a different antihuman apoB antibody with trout apoB. Remarkably, the EO6 reactivity in apoB lipoproteins was as much as 20 to 30 times higher in HCD-fed zebrafish plasma than in control plasma samples (Figure 1F). Using an anti-human apoAI antibody (Online Figure II) to trap HDL particles from plasma revealed the equally remarkable finding that EO6 immunoreactivity on apoAI particles was also 20- to 30-fold higher in the HCD plasma (Figure 1G).

Vascular Lesions in Adult Zebrafish

Next, we examined HCD-fed and control zebrafish frozen and paraffin-embedded sections stained with van Gieson stain, LipidTOX (a fluorescent stain for neutral lipids), and DAPI (a nuclear stain). In sections of partially perfused dorsal aorta, we found vascular lesions of enlarged intima that extended into the lumen of the dorsal aorta and were characterized by accumulation of lipid and cell infiltration (Figure 2, Online Figure III, and Online Table I). Such lesions are classified as fatty streaks, early lesions of developing atherosclerosis in mouse models and in humans. From a total of 9 HCD-fed fish (both males and females), 7 had lesions of 100 to 500 μm in length in a 5-mm long segment of the dorsal aorta, whereas only 1 of 9 control fish had 1 small lesion (Online Table I). The lesions were mostly found at the sites of intersegmental arteries bifurcation, where turbulent flow can be expected (Figure 2A). To detect macrophages in zebrafish vascular lesions, we used a polyclonal antibody against

Figure 1. Hypercholesterolemia and oxidized plasma lipoproteins in adult zebrafish. Five-week-old zebrafish (both male and female) were fed a 4% cholesterol-enriched (HCD) or normal (control) diet for 8 to 12 weeks. A, Female fish (confirmed by dissection) fed a control diet or HCD. B, The ratio of body weight to length (body mass index) (n=17 in each group, both males and females; no statistically significant differences). C and D, TC and triglycerides in plasma of 3-month-old zebrafish (n=11 in each group, both males and females). *P<0.001. E, Native agarose gel electrophoresis of HCD and control zebrafish plasma, stained with Fat Red. “Standard” is a human plasma (36.4% α-HDL, 18.4% pre-β [VLDL], and 45.1% β-lipoproteins [LDL]). Each “HCD” or “control” lane shows an individual zebrafish plasma sample, representative of total 35 samples. Arrows point at high-mobility bands. F and G, The EO6 immunoassay was performed with 1:200 diluted zebrafish plasma captured on a microtiter plate coated with either antihuman apoB (F) or antihuman apoAI (G) antibody. Oxidation-specific epitopes were detected with EO6 antibody (n=8). *P<0.05.
human t-plastin. Zebrafish and human t-plastin are 82% identical and 90% homologous, and in western blots the antibody stained bands of the same molecular mass in zebrafish larvae lysates and in the lysates of murine macrophages (Online Figure IV). It also stained cells in murine atherosclerotic lesions (Online Figure IV; note similar patterns of t-plastin and Mac3 stainings) and in zebrafish vascular lesions (Figure 2E), suggesting macrophage infiltration.

Vascular Lipid and Myeloid Cell Accumulation in Zebrafish Larvae

The zebrafish body is transparent during approximately 30 dpf, which would allow for a dynamic study of the processes of vascular lipid accumulation and inflammation. Thus, we explored whether HCD leads to vascular lipid accumulation in larvae. On the fifth dpf, when zebrafish larvae begin free feeding, we started the HCD, supplemented with a red fluorescent cholesteryl ester analog, and continued it for 10 days. The control diet with normal cholesterol content was also supplemented with the fluorescent cholesteryl ester analog. These 2 diets were fed to 2 groups of fli1:EGFP larvae, constitutively expressing GFP in ECs,1 which enables visualization of the vasculature. Live anesthetized zebrafish larvae were imaged using a Nikon C1-si confocal microscope. We observed that vasculature of the control and HCD-fed larvae were stained diffusely red, consistent with circulating fluorescent lipid (Figure 3A). Remarkably, only in HCD-fed larvae there were many focal areas of bright red fluorescence in blood vessels, which we interpreted as lipid accumulation in the vessel wall (either cholesteryl ester or its hydrolyzed fatty acid chain present as a free fatty acid or reesterified into triglycerides, phospholipids, or cholesteryl esters). To further confirm that the accumulation of fluorescent lipid is indeed a consequence of HCD feeding, we fed wild-type AB larvae diets with a varying concentration of cholesterol (2% to 10%). There was a dose-dependent increase in vascular accumulation of fluorescent lipid, with most reproducible results achieved at 4% cholesterol (Figure 3B). Using a 4% cholesterol diet with fli1:EGFP larvae, we observed even higher levels of fluorescent lipid accumulation than in AB larvae, on average a 5-fold increase (Figure 3C). Three-dimensional rendering demonstrated that these lipid deposits were subendothelial (Figure 3D), which would correspond to intimal lipid accumulation in mice and humans, although lipid accumulation in adventitia cannot be excluded. Although the majority of lipid deposits were observed in the caudal vein, some deposits were found in the dorsal aorta and at sites of blood vessel bifurcation as well (Figure 3E and Online Movie 1). The presence of the lipid deposits in veins can be explained by the specifics of the zebrafish circulatory system at this stage of development, when large arteries and veins connect directly rather than via a capillary network. In adult zebrafish, lesions were found only in the dorsal aorta but not in the caudal vein.

Feeding HCD to lyc:DsRed2 larvae, in which DsRed2 is expressed in monocyte/macrophages and granulocytes,21 resulted in the recruitment of red fluorescent myeloid cells to the caudal vein, within a 2-cell distance from the lumen (Figure 4), suggesting accumulation of macrophages and/or neutrophils in the vascular wall. In mammalian atherosclerosis, neutrophils are notably excluded from vascular lesions,20 and the absolute majority of myeloid cells are macrophages.

Endothelial Layer Disorganization and Permeability in HCD-Fed Larvae

Under laminar flow in noninflamed mammalian blood vessels, ECs form a regular layer with the cells oriented along the flow. At sites of turbulent flow and when the lipid deposition in the intima causes EC activation, an apparent thickness of the EC layer increases because of the loss of EC alignment, formation of large vacuole-like EC boundaries, and infiltration of macrophages, as observed in early lesions in hypercholesterolemic mice.22 In HCD-fed larvae, we observed irregularity in endothelial layer morphology of the caudal vein (Figure 5A) and apparent thickening of the EC layer in central and peripheral blood vessels (Figure 5A through 5C). Ezetimibe, an inhibitor of intestinal cholesterol absorption, added to the fish tank water during the HCD feeding period, reduced lipid deposition in the intestine and peritoneal cavity of HCD-fed zebrafish (Figure 5B, top images), which agrees with an earlier report.13 Remarkably, the ezetimibe treatment attenuated HCD-induced EC disorganization in peripheral vasculature (Figure 5B, bottom images) and reduced an apparent thickness of the EC layer in the caudal vein (Figure 5C), which likely reflects attenuated vascular inflammation.

Figure 2. Fatty streaks in the dorsal aorta of adult zebrafish. A, Dorsal aorta (DA) and caudal vein (CV) of HCD-fed zebrafish. ISA indicates intersegmental artery bifurcation from the DA; min, melanocytes accumulate around zebrafish blood vessels. B and C, Dorsal aortas of HCD-fed (B) and control (C) zebrafish; van Gieson staining. D and E, Dorsal aorta of HCD-fed zebrafish stained with LipidTOX Red (neutral lipid; merged fluorescent and bright field images) (D) and an antibody against t-plastin (macrophages) counterstained with DAPI (nuclei) (E). Scale=20 μm (A through D); 5 μm (E).
To test whether the observed EC layer disorganization resulted in the loss of its barrier function as found in mammalian atherosclerotic lesions, we injected intravenously a fluorescent dextran and observed its leakage outside the caudal vein (Figure 6 and Online Figure V). The intensity of dextran fluorescence at 5 μm from the lumen margin was 2.5-fold higher in HCD-fed larvae compared to the control. The ezetimibe treatment prevented dextran leaking from the blood vessels.

**Vascular PLA₂ Activity in Zebrafish Larvae**

Imaging of live zebrafish permits not only morphological but also functional studies. Farber et al developed a fluorescent
reporter for PLA₂ activity, PED6, and used it in the study of intestinal lipid metabolism in zebrafish. We found that in addition to intestinal and gall bladder fluorescence of PLA₂-hydrolyzed PED6, a bright emission was detected from zebrafish blood vessels following 10 days of HCD but was almost absent in the vasculature of control zebrafish (Figure 7A, top images, and 7B). To demonstrate that there was equal penetration of the fluorogenic substrate into vasculature of control and HCD-fed zebrafish, we used a phospholipid in which the fluorophore is unquenched and, thus, its fluorescence intensity is independent of being cleaved by PLA₂. There were no differences in the intensities of the control phospholipid fluorescence in the control and HCD-fed larvae (Figure 7A, bottom images, and 7B). These results suggest that fluorescent reporters can be used to study activities of enzymes involved in vascular inflammation in live zebrafish.

TLR4-Dependent Macrophage Lipid Uptake in Zebrafish Larvae

Studies of atherosclerosis in mouse models are facilitated by the availability of knockout mouse strains and engineered cell lines, but the technology for generating knockout zebrafish is not yet well established, and morpholino antisense techniques provide the gene knockdown only for first 3 to 5 dpf, before we initiate high-cholesterol feeding. To circumvent this problem, we transplanted genetically manipulated murine macrophages into the larvae that were fed HCD for 10 days before the transplantation. Because adaptive immunity in 15 to 20 dpf larvae is undeveloped, this technique allows for monitoring the function of mammalian macrophages in the environment of a zebrafish fatty streak. We applied this technique to investigate the function of TLR4 in atherogenesis.

We have previously reported that minimally oxidized LDL (mmLDL) activates macrophages in a TLR4-dependent manner. We noticed that in cell culture, mmLDL stimulated TLR4-competent J774 macrophages to accumulate lipid and that this effect was inhibited in TLR4-knockdown cells expressing TLR4-specific short hairpin (sh)RNA (Figure 8A). Remarkably, when transplanted into HCD-fed zebrafish larvae, TLR4-competent macrophages, repeatedly imaged in areas of vascular lipid deposition, accumulated fluorescent lipid at a significantly higher rate than TLR4-deficient macrophages (Figure 8B and 8C). At 24 hours after injection,
close to 30% of TLR4-competent macrophages accumulated lipid, compared to less than 10% of TLR4-deficient macrophages (Figure 8D). To confirm these results with primary cells, we used peritoneal macrophages and circulating mononuclear cells isolated from C3H mice; the C3H/HeJ mice carry the lps-d mutation in the TLR4 gene that makes the receptor nonfunctional, whereas the C3H/HeOuJ mice have normal functional TLR4. In a cell culture experiment, wild-type, but not TLR4 mutant macrophages, stimulated with mmLDL, spread (as we reported earlier24,25) and accumulated lipid (Figure 8E). Following transplantation into HCD-fed larvae, more than 40% of wild-type macrophages accumulated endogenous (dietary) lipid, compared to only 15% of TLR4 mutant cells (Figure 8F). Among the transplanted cells that have accumulated lipid, the relative amount of intracellular lipid was 3-fold lower in TLR4 mutant cells compared to wild-type macrophages (Figure 8G). These in vivo experiments suggest a novel function for TLR4 in mediating lipoprotein uptake by macrophages.

Discussion

Many researchers and clinicians agree that the treatment of atherosclerosis must begin at the earliest possible stage: the fatty streak.27 The processes that occur in the fatty streak, EC activation, monocyte recruitment, and excessive lipoprotein uptake by macrophages and the formation of proinflammatory lipid-loaded foam cells define the advancement of atherosclerosis and its complications. By feeding HCD to zebrafish, we were able to reproduce many of the processes involved in early atherogenesis. We observed hypercholesterolemia (Figure 1C), lipoprotein oxidation (Figure 1F and 1G), and fatty streak formation (Figure 2) in adult zebrafish. Moreover, in optically transparent zebrafish larvae, we observed vascular lipid deposition and myeloid cell accumulation, EC layer disorganization and increased permeability, increased PLA2 activity, and lipid accumulation by transplanted macrophages, all in live animals (Figures 3 through 8). These findings suggest that zebrafish is suitable as a model organism for studying mechanisms of the pathological processes important in early atherogenesis. However, as in any animal experimentation, using zebrafish enables modeling only certain aspects of the human disease.

Lipoprotein oxidation is a major pathogenic factor that accelerates atherogenesis.17,20 A dramatic increase in the plasma levels of the EO6-reactive oxidation-specific epitopes that we observed in HCD-fed zebrafish (Figure 1F and 1G) is very unusual for human samples or for any mammalian model of atherosclerosis. In particular, we have never observed such high levels of HDL-associated EO6 reactivity in any species. One possible explanation for these findings is that a poikilothermic fish in water at ambient temperature and at lower oxygen concentration than in the open air has developed less sophisticated antioxidative systems.28 Thus, the HCD challenge results in a higher rate of oxidation, and hence a greater accumulation of such products as oxidized phospholipids, as measured by the EO6 immunoassay. Increased PLA2 activity (Figure 7) might be a response to the elevated levels of oxidized phospholipids found in the plasma lipoproteins (Figure 1F and 1G) because such enzymes have the ability to degrade oxidized phospholipids. These data in zebrafish correlate with recent human studies showing that increasing lipoprotein-associated PLA2 activity further am-

Figure 6. HCD-induced increase in endothelial layer permeability. Experimental conditions as in Figure 5B, but no fluorescent lipid was added to the diet. Leakage of intravenously injected red fluorescent dextran (2×10⁶ Da) from the caudal vein was measured as described in Materials and Methods. Dashed lines at 5 and 20 µm from the lumen show where the fluorescence intensities were measured. Scale=50 µm. *P<0.01 (n=9 to 11 animals per group).

Figure 7. HCD-induced PLA₂ activity. A, AB larvae were fed a control diet or HCD for 10 days and then placed in a 1 µg/mL solution of PED6, a fluorogenic PLA₂ substrate, for 2 hours. Green fluorescence (hydrolyzed PED6) indicates the PLA₂ activity. In a separate set of experiments, PED6 was replaced with BODIPY-FLC5-HPC (0.67 µg/mL), a control fluorescent phospholipid whose fluorescence is independent of PLA₂ cleavage. Scale=100 µm. B, Quantification of the data presented in A (n=4).
plifies the risk of cardiovascular disease mediated by oxidized phospholipids, although we do not know yet which PLA₂ isoform in zebrafish was involved.

Studies with atherosclerosis-prone apoE<sup>−/−</sup> mice in which either TLR4 or MyD88 (a critical downstream molecule in TLRs signaling) was knocked out, demonstrated reduced atherosclerosis in the animals fed a high fat diet. Although these studies suggested a role for TLR4 in atherogenesis, the mechanisms remain obscure. Earlier, we observed that a putative endogenous ligand for TLR4, mmLDL, induced extensive membrane ruffling in macrophages and cell spreading, associated with intracellular vacuolization. Based on these findings, we hypothesized that the TLR4-mediated cytoskeletal rearrangements and liquid phase uptake may quantitatively increase the rate of lipoprotein uptake by macrophages and thus accelerate foam cell formation. We tested this hypothesis using the zebrafish model. Because lipoprotein oxidation occurs in vivo in HCD-fed zebrafish (Figure 1F and 1G), we expected that an in vivo generated zebrafish analog of mmLDL may induce TLR4-dependent lipid uptake. Indeed, we observed that murine macrophages transplanted into HCD-fed larvae were fed for 10 days a HCD supplemented with red fluorescent lipid. Repetitive images of the same cells in live fish were captured (red, macrophages; blue and white, fluorescent lipid; green, ECs). Scale=10 μm. From the data collected in experiments in B, the time courses of lipid uptake by individual transplanted macrophages were measured for control and TLR4 KD J774 cells (n=25 to 30 cells for each time point; total of 10 animals imaged). *P<0.01. D, In a separate set of experiments, the percentage of transplanted macrophages that accumulated fluorescent lipid 24 hours after injection into HCD-fed larvae was determined (n=9). E, In a cell culture experiment, the uptake of Alexa Fluor 488-labeled native LDL (150 μg/mL) was stimulated by nonlabeled mmLDL (50 μg/mL) for 1 hour in wild-type and TLR4 mutant primary macrophages harvested from C3H mice. Red indicates F-actin; green and white, Alexa488-LDL. Scale=5 μm. F, In a zebrafish transplant experiment, performed as in B, a percentage of transplanted primary macrophages that accumulated lipid 2 hours post injection into HCD-fed larvae was determined for wild-type and TLR4 mutant primary macrophages (n=5 animals per group; total of 234 wild-type and 125 TLR4 mutant cells were counted). G, Integrated intensities of intracellular fluorescent lipid in only those transplanted primary macrophages that were counted in F (n=26 for wild-type and n=11 for TLR4 mutant cells; not all positive cells were suitable for quantification because of their position or image quality).
transparency of zebrafish larvae provides the opportunity to observe specific processes in the vascular wall repeatedly over time in a live animal. Adult zebrafish can be used to study in vivo lipoprotein oxidation and its attendant biological responses. Economic colony maintenance, ease of genetic manipulation, fast maturation, short feeding periods, and a simple method of drug administration make the zebrafish model particularly attractive for studies of vascular lipid accumulation and inflammation.

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Disclosures
None.

References
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SUPPLEMENT MATERIAL

Stoletov et al. Vascular lipid accumulation, lipoprotein oxidation and macrophage lipid uptake in hypercholesterolemic zebrafish

In this Supplement: 1. Online Table I 2. Online Figures I through V 3. Legend for Online Movie I 4. Complete Material and Methods section

Online Table I. Dorsal aorta lesions in control and HCD-fed adult zebrafish. Segments of dorsal aorta were cryo-sectioned as described in Methods and examined under a bright field microscope. Lesions were defined as areas of enlarged intima that extended into the lumen. Examples of such lesions are shown in Figure 2 and Online Figure III. An approximate length of the lesions was calculated as a number of consecutive sections that contained lesions multiplied by the section thickness (10 μm).

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Online Figure I. Total cholesterol (TC) and triglycerides (TG) levels in plasma of HCD-fed zebrafish of various age and gender. TC and TG levels were measured in individual plasma samples of 3 month and older zebrafish using standard automated assays. Plasma collected from 1.5 month old larvae were pooled from three-to-five animals in order to obtain a sufficient sample volume.
Online Figure II. Western blot of zebrafish and human plasma with polyclonal antibodies against human apoB and apoAI. SDS-PAGE and western blot were performed according to a standard protocol. Primary antibodies were generated earlier in our laboratory by immunizing guinea pigs with human proteins.
Online Figure III. Examples of vascular lesions in the dorsal aorta of HCD-fed adult zebrafish. A – C, Cross-sections of partially perfused zebrafish dorsal aorta. Bright field and bright field merged with DAPI (blue nuclei) images. D, Bright field and LipidTox Red staining of the dorsal aorta of HCD-fed zebrafish. Upper panels show a vascular lesion, and lower panels represent a non-affected segment of the same aorta. E, Dorsal aorta (DA) and caudal vein (CV) of a zebrafish fed control diet. Compare with the section in Figure 2A. Scale, 20 μm.
Online Figure IV. Anti-human L-plastin antibody as a reagent for zebrafish macrophage detection. A, Consecutive sections of murine lesions in aortic origin stained with antibodies against human L-plastin and mouse Mac3, a common macrophage marker. B, Consecutive sections of murine lesions stained with an anti-human L-plastin antibody and the antibody plus a specific blocking peptide (BP); fluorescent detection. C, The same antibody staining cells (white) in a zebrafish lesion; nuclei counterstained with DAPI (blue). D, The same antibody and the blocking peptide as in B staining zebrafish vascular lesions. E, The same antibody and the peptide as in B staining murine J774 macrophages in cell culture. F, Western blot of the whole body zebrafish larvae lysates and the lysates of J774 cells, stained with the same antibody and the blocking peptide as in B. Arrows indicate bands that show specific L-plastin staining, i.e. their antibody binding was blocked by the blocking peptide. Note that the L-plastin band in zebrafish is weaker than in murine macrophages because detected in the lysate of the whole larvae body, not in isolated zebrafish macrophages.
**Online Figure V. Dextran leak from the caudal vein.** Conditions as in Figure 6. Red fluorescence of dextran is shown in black and white to better visualize extravascular fluorescence in the HCD-fed animal. Scale, 100 μm.

**Online Movie I. Vascular lipid accumulation in HCD-fed larvae.** This rotation video was derived from the 3D reconstruction image an *en face* projection of which is shown in Figure 3E in the text. Note red deposits of accumulated lipid adjacent to the outer surface of endothelial cells (green EGFP fluorescence) of both the dorsal aorta (upper blood vessel) and the caudal vein (lower, wider blood vessel).
**Zebrafish.** Tg(fli1:EGFP) zebrafish, in which endothelial cells express EGFP, and wild type AB zebrafish stocks were purchased from Zebrafish International Resource Center. Tg(lyz:DsRed2), in which monocyte/macrophages and granulocytes express DsRed2, was generated as described. Zebrafish maintenance and procedures were approved by the UCSD institutional animal care and use committee. Larvae zebrafish were imaged anesthetized (short exposure to 0.02% Tricaine), then awakened in fresh water and released to the fish tank. Procedures with adult zebrafish were performed postmortem. The fish were euthanized by a prolonged exposure to 0.02% Tricaine.

**Feeding.** During experimental feeding period, a control diet for adult zebrafish was salmon starter (Aquaneering). A high-cholesterol diet (HCD) was made by soaking salmon starter in a diethyl ether solution of cholesterol (Sigma) to achieve a content of 4% (w/w) cholesterol in the food after ether evaporation. HCD and control diets were fed to adult zebrafish for periods of 8-12 weeks. Similarly, larvae were fed artificial artemia (Azoo) that was enriched with 2-10% cholesterol using the same procedure. In most experiments, a 4% cholesterol diet was used. For the purposes of studying vascular lipid accumulation in larvae, both control and HCD food were supplemented with 10 μg/g of red fluorescent cholesteryl ester analog (cholesteryl 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoate, or cholesteryl BODIPY® 576/589 C11 from Invitrogen) by soaking the food in a diethyl ether solution of cholesteryl BODIPY® 576/589 C11. HCD and control diets were fed to larvae zebrafish for the periods of 7-14 days.

**Lipid and lipoprotein analyses.** 2 μl blood was drawn from the heart and immediately diluted in 9 μl of ice-cold PBS-EDTA (Versene from Invitrogen). After centrifugation, 1:10 diluted plasma was collected and used for analysis. Total cholesterol (TC) and triglycerides (TG) in plasma were measured using automated enzymatic assays (Roche Diagnostics and Equal Diagnostics). Plasma lipoprotein profiles were analyzed in native agarose gel electrophoresis with Fat Red staining (Helena Laboratories). Lipotrol was used as a standard and consisted of human lipoproteins: 36.4% alpha- (HDL), 18.4% pre-beta- (VLDL) and 45.1% beta-lipoproteins (LDL). To analyze lipoprotein oxidation, 1:200 diluted zebrafish plasma was captured on a microtiter plate coated with either anti-human apoB or anti-human apoAI antibody (guinea pig polyclonals developed in our laboratory). Oxidation-specific epitopes were then detected with EO6 monoclonal antibody (developed in our laboratory). On average, we were able to perform two different assays with each individual plasma sample obtained from adult zebrafish. Blood drawn from 40-45 dpf larvae were pooled to performed TC and TG measurements.

**Histology.** An euthanized adult zebrafish was immersed into ice-cold PBS containing 10 mM EDTA, and a trunk/tail section including dorsal and ventral fins was isolated. To remove blood, the trunk/tail segment was gently massaged in several changes of cold PBS/EDTA, followed by a 1 hour incubation with red blood cells lysis buffer at room temperature. The segment was subsequently fixed in 10% Zn-formalin for 2 days at 4°C, decalcified in 0.5 M EDTA/PBS for 7 days at 4°C, gradually equilibrated with 25% sucrose in PBS ending in 100% OCT Compound (Tissue-Tek), and then frozen using dry ice and isopropanol. Ten μm serial sections were collected using a Leica CM1900 cryostat and kept at -80°C. Initial morphological evaluation of non-stained sections was performed using a bright field microscope. Sections containing lesions in the dorsal aorta (intimal thickening resembling fatty streaks in human and mouse aorta) were air-dried, rinsed with PBS and used for staining. An estimate of lesion occurrence in control and HCD-fed zebrafish is in Table S1. Alternatively, tissues were paraffin-embedded, sectioned, deparaffinized and used for staining. Van Gieson’s stain was used to assess lesion morphology. Lipid accumulation in the dorsal aorta was visualized in sections stained with a LipidTOX™ Red neutral lipid stain (Invitrogen) for 60 min (1:1000) and mounted with Prolong® Gold mounting. 
media containing DAPI for nuclear staining (Invitrogen). Macrophages in zebrafish histological sections were identified with an anti-human L-plastin polyclonal antibodies; the specificity of staining was controlled by adding a blocking peptide (both from Santa Cruz Biotechnology). We determined that this antibody stains bands of the same molecular mass in western blots of zebrafish larvae lysates and the lysates of murine macrophages. The L-plastin antibody also stained mouse atherosclerotic lesions, and the pattern of the staining was similar to the staining by an antibody to Mac3 (BD Biosciences, PE-conjugated), a macrophage marker commonly used in atherosclerosis studies. Images were captured with a Leica CTR5000 fluorescent microscope equipped with a Leica DC500 camera or with a Delta Vision digital microscopic system (Applied Precision). In some figures, fluorescent images were superimposed onto phase contrast images using Photoshop CS2 software (Adobe).

**In vivo microscopy.** For in vivo confocal microscopy anaesthetized fish larvae were housed in a sealed, temperature controlled chamber (20/20 Technology) in a small drop of Tricaine (0.003%) containing water. A Nikon C1-si confocal microscope was used in either regular or spectral acquisition modes. The regular one- or two-cannel modes were used to detect fluorescence of one or two fluorophores in the same larva, such as in Figures 3-7. In the green channel, excitation was 488 nm for EGFP, hydrolyzed PED6 and BODIPY®- FLC5-HPC, and in the red channel, 561 nm for BODIPY® 576/589 C11. The spectral mode was used to detect three fluorophores in the same larva, such as in Figure 8. Spectral images were obtained in a single scan with a 32-channel PMT detector coupled with the built-in unmixing software. Spectral characteristics of single fluorophores (EGFP – endothelial cells, CellTracker Orange – macrophages, and BODIPY 576/589 C11 – lipid) in larvae were obtained and an unmixing algorithm was designed to separate the three colors in the same sample from a spectral image. For presentation purposes, the colors in Figure 8 were re-coded to display orange (macrophages) as red, and red (lipid) as blue. For the purposes of 3D rendering, 0.5-2 µm step z-stacks (512 x 512 focal planes, 20-100 µm in depth) were acquired over a 1-3 min period using 10x, 20x or 63x objectives. Images were 3D rendered and analyzed using Imaris® software (Bitplane). All 3D reconstructions were done with the same threshold settings.

**Quantifying vascular lipid accumulation in larvae.** *fli1:EGFP* larvae at 5-7 dpf were fed control or high-cholesterol diets, both supplemented with 10 µg/g cholesteryl BODIPY® 576/589 C11 for 10 days. The larvae were anaesthetized with 0.02% Tricaine and optical sections in the trunk/tail area were collected with a 20x or 63x objective. The captured images of control and HCD-fed fish were first exported as slice images into TIFF format and then BODIPY® 576/589 intensities were quantified using VisionWorks®LS Image Analysis software (UVP). A threshold for the BODIPY® 576/589 intensity was adjusted to be above the diffuse signal of the fluorophore in the lumen.

**Quantifying numbers of myeloid cells associated with the caudal vein.** *lyz:DsRed2* larvae at 5-7 dpf were fed control or high-cholesterol diets for 10 days. The larvae were anaesthetized with 0.02% Tricaine and images (red fluorescence and bright field) in the trunk/tail area were collected with a 20x objective. The location of the caudal vein was determine by the blood flow. Numbers of red fluorescent cells that were found within 50 µm from the lumen of the caudal vein were determined in a 640 µm long segment of trunk/tail.

**Quantifying an apparent thickness of the EC layer.** *fli1:EGFP* larvae were fed control or high-cholesterol diets, both supplemented with 10 µg/g cholesteryl BODIPY® 576/589 C11 for 10 days. One group of the HCD-fed fish was kept in the system water supplemented with 40 µg/ml ezetimibe for the whole feeding period. Optical sections through the whole width of the caudal vein were captured using a 63x objective. GFP fluorescence from the EC layer was detected in the green channel, and diffuse cholesteryl BODIPY® 576/589 C11 fluorescence in the caudal vein lumen was detected in the red channel. In this set of experiments, unlike in
experiments quantifying vascular lipid deposition, a BODIPY® 576/589 intensity threshold was lower to measure the volume of the lumen by quantifying the fluorophore intensity in circulation rather than in the vessel wall. Two 3D contour surfaces were built with Imaris software (see drawing on the left): Volume A delineating the outer surface of the EC layer (green channel; see also Figures 3D and 4A in the text showing examples of Imaris-built contours) and Volume B delineating the surface of the lumen (red channel). Dividing Volume A and Volume B by the length of the blood vessel section (c) gave us the areas of two concentric circles A (green+red) and B (red only). An average thickness of the EC layer (x) was calculated by subtracting Radius B from Radius A. Although the actual shape of rendered blood vessels in a cross section was elliptic, we used a circular approximation to calculate the EC layer thickness averaged over the blood vessel circumference.

**Quantifying EC layer permeability.** For intravenous dextran injections we used the protocol developed in our earlier work\(^4\). In brief, at the end of the feeding period, zebrafish were anesthetized with 0.02% tricaine, and injected with TexasRed-conjugated 2×10^6Da dextran (0.5 mg/ml; Invitrogen) into the cardinal vein using an FemtoJet microinjector equipped with a 0.75-mm diameter borosilicate glass needle (length, 50 mm; diameter of the needle opening, 5 μm). Injected fish were washed once with system water and transferred into a Petri dish with system water for 1 hour. Fish were imaged 1 hour later using a Nikon C1si microscope. Using ImageJ software, intensities of the dextran fluorescence were quantified inside the lumen of the caudal vein and outside, at 5, 10, 15 and 20 μm from the lumen, and averaged over 300 μm long segments. The dextran leakage was determined as a ratio of fluorescence intensity outside the lumen to the intensity inside the lumen. This method of normalization permits to account for the variations in the amounts of dextran injected.

**Quantifying vascular PLA2 activity in larvae.** AB larvae at 5-7 dpf were fed control or high-cholesterol diets, not supplemented with any fluorescent additive. At the end of feeding period, live larvae were soaked for 2 hours in either N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine (PED6, 1 μg/ml, from Invitrogen) or 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY®-FLC5-HPC, 0.67 μg/ml – equal molar concentration with PED6, from Invitrogen) in 1 ml of system water. The larvae were briefly washed, anesthetized and imaged as described above. The fluorescence intensity of PLA2-hydrolyzed PED6 corresponds to the PLA2 activity\(^5\), while the PLA2-independent fluorescence intensity of BODIPY®-FLC5-HPC was used as an indicator of equal substrate penetration into the vasculature of control and HCD-fed larvae. The images were first exported as slice images into TIFF format and then quantified using VisionWorks®LS Image Analysis software (UVP).

**Cell transplant and quantifying cellular lipid accumulation.** To monitor in vivo lipid accumulation by macrophages with altered expression of TLR4, we transplanted murine macrophages into fltl:EGFP zebrafish larvae, which had been fed HCD supplemented with 10 μg/g BODIPY® 576/589 C11 for 10 days and had vascular lipid deposits (red fluorescence) already formed. In one set of experiments, the transplanted macrophages were murine macrophage-like cell line J774 expressing TLR4-specific or scrambled shRNA (see below). In another set of experiments, we used circulating mononuclear leukocytes isolated on Histopaque-1077 (Sigma) from the blood of C3H/HeJ mice (TLR4-deficient) or C3H/HeOuJ mice (TLR4-
competent). Both mouse strains were purchased from the Jackson Laboratory, and the mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. Macrophages were labeled with CellTracker Orange (Invitrogen) according to the manufacturer’s protocol. The cells were washed and resuspended in 0.9×PBS supplemented with 1% FBS, 0.3 U/µl Heparin (Baxter) and 0.1 U/µl DNase (Qiagen) to a final density of 10^4/µl. Injections were performed using a FemtoJet microinjector (Eppendorf) and borosilicate glass needles (diameter of the needle opening = 20 µm). Five to 10 µl of a cell suspension was back loaded into the capillary and 50-100 cells were injected into larvae in the area between dorsal aorta and caudal vein (murine cells injected intravenously into larvae were trapped in the gills capillaries and did not circulate). Anesthetized larvae were imaged at 0.5, 2, 6 and 24 hours post injection and kept in system water at 35ºC in between imaging sessions. Five to 10 cells per fish that localized close to blood vessels (green EGFP fluorescence of endothelial cell) were imaged in the spectral mode with subsequent unmixing as described above and quantification using VisionWorks®LS Image Analysis software (UVP).

**Generation of TLR4-deficient J774 macrophages.** Predesigned TLR4-specific and control scrambled shRNA vectors, carrying the neomycin resistance gene, were purchased from SuperArray. To ensure the specificity of a knockdown, two different shRNA sequences were used (TCCCTGATGACATTCCTTCTT and GCCGTTGGTGTATCTTTGAAT). J774 macrophages were transfected with the shRNA vectors using Lipofectamin 2000 (Invitrogen). After 24 hours, cells were split into dishes with complete medium containing 0.8 mg/ml G418, and knockdown clones were selected for 3-4 weeks. The TLR4 knockdown was confirmed by testing its surface expression with a rat anti-mouse TLR4/MD2 antibody and an IgG2a isotype control (BD Pharmingen) in a FACS analysis. The surface expression of TLR4 was reduced by 60% in TLR4 KD cells compared to the control cells expressing scrambled shRNA.

**LDL isolation and modification.** LDL (density=1.019-1.063g/ml) was isolated from plasma of normolipidemic donors by sequential ultracentrifugation. Native and modified LDL preparations were tested for possible endotoxin contamination using a LAL kit (Cambrex). LDL preparations with LPS content higher than 50 pg/mg protein (i.e. 2.5 – 7.5 pg/ml in cell culture experiments) were discarded. To produce mmLDL, we incubated 50 µg/ml of LDL in serum-free DMEM for 18 hours with murine fibroblast cells overexpressing 15-lipoxygenase 6,7. We previously documented that this generates a minimally oxidized LDL, i.e. it binds to native LDL receptors but not to scavenger receptors. mmLDL contains early lipid peroxidation products, but it does not contain any measurable thiobarbituric acid reactive substances (TBARS) and does not bind to monoclonal antibody EO6 (specific to oxidized PC-containing phospholipids or oxidized phospholipid-protein adducts) above the level seen in native, non-oxidized LDL.

**Statistics.** Data in graphs are presented as mean ± standard error. Statistical differences between experimental groups were evaluated by one-way ANOVA test. Values of p < 0.05 were considered statistically significant.

References


