The RXR Agonist Bexarotene Improves Cholesterol Homeostasis and Inhibits Atherosclerosis Progression in a Mouse Model of Mixed Dyslipidemia

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Objective—The activity of the antitumoral agent bexarotene (Targetretin, Bexarotene) depends on its binding to the nuclear retinoid-X receptor (RXR) and subsequent transcriptional regulation of target genes. Through RXR activation, bexarotene may modulate numerous metabolic pathways involved in atherosclerosis. Here, we investigated the effect of bexarotene on atherosclerosis progression in a dyslipidemic murine model, the human apolipoprotein E2 knockin mouse, that develops essentially macrophage-laden lesions.

Methods and Results—Atherosclerotic lesions together with different metabolic pathways involved in atherosclerosis were investigated in mice treated or not with bexarotene. Bexarotene protects from atherosclerosis development in mice, at least in part by improving the circulating cholesterol distribution profile likely via a marked decrease of dietary cholesterol absorption caused by modulation of intestinal expression of genes recently identified as major players in this process, Niemann-Pick-C1-Like1 (NPC1L1) and CD13. This atheroprotection appears despite a strong hypertriglyceridemia. Moreover, bexarotene treatment only modestly modulates inflammatory gene expression in the vascular wall, but markedly enhanced the capacity of macrophages to efflux cellular lipids.

Conclusion—These data provide evidence of a favorable pharmacological effect of bexarotene on atherosclerosis despite the induction of hypertriglyceridemia, likely via a beneficial action on intestinal absorption and macrophage efflux. (Arterioscler Thromb Vasc Biol. 2006;26:2731-2737.)

Key Words: atherosclerosis | cholesterol homeostasis | intestinal cholesterol absorption | retinoid | triglycerides

Bexarotene [Targetretin, 4-[1-(5,6,7,8-tetrahydro-3,5,8,8-pentamethyl-2-naphthalenyl) ethenyl] benzoic acid] is an antitumoral agent used as chemotherapy in the treatment of cutaneous T-cell lymphoma. Bexarotene is currently being evaluated for the treatment of other cancers and psoriasis. Thus, bexarotene is both an element of the current antitumoral therapeutic arsenal and a molecule with emerging and promising effects in various pathologies.

Atherosclerosis is a complex inflammatory pathology of the vascular wall, precipitated by systemic factors, such as qualitative or quantitative abnormalities of circulating lipids and lipoproteins. Blood lipid concentrations reflect an equilibrium between absorption of dietary lipids in the small intestine, production after endogenous synthesis in the liver, and removal by different peripheral tissues and the liver. In pathological conditions, circulating atherogenic lipoproteins can be taken up by macrophages in the vascular wall, thus initiating an inflammatory process leading to a progressive evolution of atherosclerosis. Through the action of locally produced cytokines and other inflammatory proteins leading to cell migration and proliferation, the vascular wall is continuously remodeled. Atherosclerosis progressively evolves from the simple fatty streak to advanced atherosclerotic plaques, which may ultimately lead to plaque rupture and thrombus formation.

The pharmacological responses to bexarotene originate from the transcriptional control of gene programs via activation of a member of the nuclear receptor superfamily, the retinoid-X receptor (RXR). As other nuclear receptors, RXR acts as a transcription factor that, on activation by binding of a specific ligand, binds to gene regulatory DNA sequences and subsequently modulates the transcription of target genes. A growing number of studies have reported effects of RXR ligands on plasma lipid and apolipoprotein concentrations, cell migration, proliferation, apoptosis, matrix remodeling, and inflammation, all of which impinge on atherogenesis.
A beneficial effect on atherogenesis in vivo has been reported only once, with the rexinoid LG100364, a molecule that was never further developed, and thus has no clinical applications, in the apolipoprotein E (apoE) knockout (apoE-KO) mouse. In the present report, we studied the apoE2-KI mouse model, which differs from the apoE-KO mouse model, because it rather develops a mixed dyslipidemia combining hypertriglyceridemia and hypercholesterolemia as frequently found in humans, whereas apoE-KO mice are characterized by an isolated hypercholesterolemia. Furthermore, apoE2-KI mice appear a better pharmacological model to test agonists of nuclear receptors than apoE2-KO mice. Because the concept of selective nuclear receptor modulator (SNRM) agonists, which postulates that different agonists of a nuclear receptor lead to overlapping but also distinct biological effects, also pertains to RXR modulators and because apoE2-KI mice display hypertriglyceridemia that is exacerbated by rexinoid treatment as observed in humans treated with rexinoids, we decided to study the effects of bexarotene, a rexinoid used in humans, on atherosclerosis and related metabolic pathways in the apoE2-KI model.

We show here that bexarotene protects apoE2-KI mice from atherosclerotic lesion development, despite a marked increase in triglycerides, at least in part, by decreasing the atherogenic cholesterol-containing lipoprotein fraction likely due to a marked decrease of dietary cholesterol absorption in relation to decreased intestinal expression of Niemann-Pick C1-Like1 (NPC1L1) and CD13. Bexarotene treatment only modestly modulates the expression of inflammatory genes in the vascular wall, but enhanced the capacity of macrophages to efflux cellular lipids. Thus pharmacological atheroprotection can be obtained despite triglyceride elevation when atherogenic lipoprotein concentrations decrease.

Materials and Methods

Animals and Treatment

Homozygous female human apoE2-KI mice on a C57BL6 genetic background were used. The animal experiments were performed according to the institutional guidelines. Mice were euthanized by cervical dislocation.

Bexarotene was synthesized in the Laboratoire de Chimie Pharmacutique (Faculté des Sciences Pharmaceutiques, Université de Lille 2, France).

In three different experiments, 2 separate groups of apoE2-KI mice were matched for age. Doses of bexarotene, within the range of doses previously used in rodents, efficient on lipid parameters and nontoxic during the treatment duration were used. Twenty-four mice (n=12/group) aged 7 to 10 weeks were fed a Western-style diet supplemented (BEXA) or not (CONT) with 0.018% bexarotene for 11 weeks. A, Oil-Red-O staining of atherosclerotic lesions in aortas of control (○) and treated (●) mice. Bars=0.5 mm. Arrows indicate traces of remaining Oil-Red-O staining parts.

For these experiments, tissues and cells were frozen in liquid nitrogen (LN2) and stored at −80°C until mRNA analysis.

Sixteen chow diet fed mice (n=8/group) aged 6 months were dosed once daily first with bexarotene (300 mpk) or vehicle (carboxymethylcellulose 1%/PEG 400/Tween, 90/9.95/0.05, by volume) for 5 days, and then with a single dose of [14C]cholesterol (1 μCi) and [3H]sitostanol (2 μCi) in 150 μL of olive oil. The mice were subsequently dosed once daily with bexarotene or vehicle for 4 additional days, totaling 9 consecutive treatment days. Feces were collected during the 4 last days to calculate intestinal cholesterol absorption (see http://atvb.ahajournals.org).

For expanded Materials and Methods used in this article, please see http://atvb.ahajournals.org.

Results

Bexarotene Decreases Atherosclerosis Development in ApoE2-KI Mice

The apoE2-KI mice were fed a Western diet with or without bexarotene for 11 weeks. The treated group gained weight to a comparable extent as the control group, indicating absence of toxicity (data not shown).

Bexarotene-treated mice displayed a marked decrease in atherosclerotic lesion areas as compared with control mice (median 0.028 versus 0.129 mm2, P<0.001) (Figure 1A). Microphotographs representative of treated and control mice (Figure 1B) show the decrease of lipid-stained surfaces induced by treatment. Moreover, as revealed by MOMA-2-
specific staining of the lesions, macrophage content was reduced on bexarotene treatment and colocalized with Oil-Red-O–stained areas (data not shown).

**Bexarotene Increases Plasma Triglyceride Concentrations in ApoE2-KI Mice**

Compared with controls, bexarotene-treated mice showed a marked increase in plasma triglyceride concentrations (+50%, \(P<0.001\)) (Figure 2A). The triglyceride distribution profile showed that triglycerides were associated with very-low-density lipoprotein (VLDL) (data not shown). To analyze the mechanisms involved in this increase, the hepatic expression of proteins controlling triglyceride metabolism were measured. Stearyl-coenzyme A (CoA) desaturase 1 (SCD1) and fatty acid synthase (FAS) are lipogenic enzymes, whereas angiopoietin-like 3 (Angptl3) is implicated in triglyceride catabolism. The expression of genes encoding SCD1 and FAS was strongly increased in livers of treated mice as compared with controls (\(P<0.001\)), whereas Angptl3 mRNA was only slightly increased (\(P<0.05\)) (Figure 2B).

Because the activity of SCD1 can be evaluated by determining the ratio of oleic acid to stearic acid (desaturation index; C18:1/C18:0),\(^{12}\) the fatty acid composition of cholesteryl esters in plasma was measured (see http://atvb.ahajournals.org, Table II). Expressed as percentage of total fatty acid mass in cholesteryl esters, saturated fatty acids with 16 and 18 carbon atoms were significantly decreased (\(P<0.001\)) in treated mice, whereas monounsaturated fatty acids with the same number of carbons were unchanged, leading to a significant increase of the ratios C18:1/C18:0 and C16:1/C16:0, indicative of an enhanced SCD1 activity.

**Bexarotene Decreases Atherogenic Cholesterol-Containing Lipoproteins in ApoE2-KI Mice**

Bexarotene-treated mice exhibited a reduction in total cholesterol levels (\(P<0.05\)), which was entirely caused by reduced non– high-density lipoprotein (HDL) cholesterol concentrations (\(P<0.01\)), whereas HDL cholesterol levels were comparable in treated and control mice (Figure 3A). Cholesterol distribution profile analysis in control mice (Figure 3B) clearly showed that in the apoE2-KI mouse model, cholesterol is mainly transported by VLDL, intermediary-density lipoprotein (IDL), and low-density lipoprotein (LDL), whereas the HDL fraction represents only \(\approx20\%\) of total cholesterol as previously observed.\(^{13}\) Treatment with bexarotene led to a decrease in non-HDL cholesterol exclusively by decreasing the IDL–LDL fraction without any change in the HDL fraction (Figure 3B). The reduction of the atherogenic cholesterol-rich remnant particles in treated mice was associated with reduced plasma concentrations of its main protein component, apoB (\(P<0.01\)) (Figure 3C), but hepatic apoB mRNA levels were unchanged (Figure 3D). Because marked changes were observed in the cholesterol distribution profile in treated mice, the mechanisms that could explain these modifications were further analyzed by measuring the hepatic expression of genes controlling cholesterol homeostasis. While LDL receptor mRNA levels were markedly enhanced (\(P<0.001\)) in treated mice as compared with controls (Figure

![Figure 2. Bexarotene increases plasma triglycerides in apoE2-KI mice.](http://atvb.ahajournals.org/)

Female apoE2-KI mice were fed a Western-style diet supplemented (BEXA) or not (CONT) with 0.018% bexarotene (n=12/group) for 11 weeks. Mean±SD. *\(P<0.05\), **\(P<0.01\) vs controls. A, Plasma triglyceride concentrations in control (white bars) and treated (black bars) mice. B, Hepatic SCD1, FAS and Angptl3 mRNA levels. Data are expressed as percentages, arbitrary values of 100% being attributed to the control group.
3E), 3-hydroxy-3-methylglutaryl (HMG)-CoA-synthase (HMG-CoA-S) gene expression was not altered (Figure 3F), suggesting that hepatic removal of LDL from circulation could be enhanced by treatment without concomitant changes in cholesterol synthesis.

**Bexarotene Decreases Intestinal Cholesterol Absorption and Modulates Expression of Intestinal Genes in ApoE2-KI Mice**

To gain insight in the mechanisms underlying the reduced LDL/IDL levels in treated mice, intestinal cholesterol absorption was determined. Bexarotene treatment led to a 64% reduction of cholesterol absorption efficiency ($P<0.001$) (Figure 4). Since NPC1L1 and CD13 have recently been identified as critical components of the intestinal cholesterol absorption machinery, intestinal mRNA levels of these genes were measured. NPC1L1 and CD13 mRNA levels were significantly decreased in both duodenum and jejunum of bexarotene-treated mice as compared with controls (Figure 5). In addition, as ABCA1 has been shown to be implicated in intestinal cholesterol metabolism, its expression was also measured. ABCA1 mRNA levels were significantly lower in treated mice than in controls (Figure 5). Comparable decreases both in cholesterol absorption and expression of these genes were observed in mice fed a Western diet for 11 weeks (data not shown).

**Bexarotene Improves Macrophage Lipid Homeostasis In Vivo and In Vitro, and Induces ABCA1 and ABCG1 Expression**

The efficiency of plasma from treated and control mice to promote cholesterol efflux ex vivo in Fu5AH hepatoma cells was comparable (30.5 ± 4.7% versus 27.1 ± 6.2%, treated versus controls, $P=0.118$).
Interestingly, mice fed a Western diet displayed an increased number of Oil-Red-O–stained peritoneal macrophages (73% of total cells) compared with mice fed a chow diet (14% of total cells), and bexarotene treatment strongly reduced (33% of total cells) the Western diet-induced lipid-loading of these peritoneal macrophages (Figure 6A). To further analyze the mechanism, cholesterol-loaded peritoneal macrophages isolated from apoE2-KI mice and in vitro treated with bexarotene were incubated with either apoA-I or HDL as cholesterol acceptors, and cellular cholesterol efflux was measured. The cholesterol efflux was significantly higher in bexarotene-treated macrophages whatever the acceptor (P < 0.05) (Figure 6B). Because ABCA1 and ABCG1 are the main cholesterol transporters mediating apoA-I–dependent and HDL-dependent cholesterol efflux pathways, respectively, the expression of these transporters was compared in the aortic sinus of bexarotene-treated and control mice. The mRNA levels of both proteins were significantly induced by bexarotene treatment (P < 0.01) (Figure 6C).

**Bexarotene Only Modestly Modulates Gene Expression of Cytokines in the Aortic Sinus of ApoE2-KI Mice**

The expression of genes encoding several molecules implicated in the inflammatory process was measured in the aortic sinus of 6-month-old chow-fed female mice after a 14-day treatment with bexarotene. At this age, female apoE2-KI mice display large macrophage-laden lesions in the aortic sinus (personal unpublished results), which allows to study gene expression in the context of inflammatory protein producing cells. Monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) mRNA levels were minimally altered in bexarotene-treated mice (MCP-1 161 ± 75 versus 100 ± 28, treated versus controls, P < 0.05; macrophage colony-stimulating factor 72 ± 18 versus 100 ± 17, treated versus controls, P < 0.05). Expression of other genes studied, IL-6, IL-12, tumor necrosis factor-α, cyclo-oxygenase 2, tissue factor, and vascular cellular adhesion molecule-1, was not significantly changed by treatment (supplemental Figure I, available online at http://atvb.ahajournals.org).

**Discussion**

In the present report, we show that bexarotene, a retinoid used in humans, inhibits atherogenesis in the apoE2-KI mouse. Bexarotene treatment induces effects both on systemic plasma lipid parameters and on macrophage lipid homeostasis. Bexarotene likely exerts atheroprotection in apoE2-KI mice by lowering circulating atherogenic cholesterol-containing lipoproteins. Interestingly, the pronounced effect of bexarotene treatment on

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*Figure 6. Bexarotene increases macrophage lipid efflux. A, Representative micrographs of in vivo lipid-loaded peritoneal macrophages. Female apoE2-KI mice were fed a chow diet (left), a Western diet (middle), or a Western diet supplemented with bexarotene for 12 days (right). Peritoneal macrophages were recruited using thioglycollate and stained ex vivo by Oil-Red-O. Bar = 50 μm. B, Apo A-I and HDL mediated-cholesterol efflux in peritoneal macrophages obtained from apoE2-KI mice. Macrophages were in vitro treated with bexarotene (1 μmol/L) for 24 hours and cholesterol efflux measured. Results are expressed as fold induction as compared with controls. Mean ± SD. *P < 0.05 vs controls. C, ABCA1 and ABCG1 gene expression in the aortic sinus. Female apoE2-KI mice fed a chow diet were treated with bexarotene (300 μg/kg) or vehicle for 12 days (n = 8/group). Aortic sinuses were removed from individual mice, RNA extracted, and gene expression analyzed. Data are expressed as percentages, arbitrary values of 100% being attributed to the control group. Mean ± SD. **P < 0.01 vs controls.*
plasma cholesterol concentrations coincides with a decrease in intestinal cholesterol absorption, which is associated with a reduced intestinal gene expression of NPC1L1 and CD13. Indeed, NPC1L1 has been recently identified as a target of the hypocholesterolemic drug ezetimibe through regulating intestinal cholest erol absorption.14–18 Thus, inhibiting NPC1L1 activity by ezetimibe in apoE-KO mice14 or gene deletion in bexarotene in apoE2-KI mice leads to a decreased susceptibility to atherosclerosis. CD13 has been identified as a molecule potentially implicated in cholesterol absorption19 and could also be a molecular target of ezetimibe.9 Interestingly, bexarotene decreased the expression of ABCA1 in the intestine. This result differs from observation with an other rexinoid, LG268, showing an increased ABCA1 expression in the intestine associated with a decreased intestinal cholesterol absorption.20 However, the potential effect of ABCA1 in cholesterol absorption has been highly debated21–23 and a recent study24 showed its implication in intestinal HDL biogenesis.

One level of complexity of RXR biology relates to its ability to activate transcription as an obligate partner of heterodimerization with many nuclear receptors. Thus, bexarotene activation of RXR could elicit responses of the permissive heterodimers and thus modulate cholesterol absorption. The question addressing which partner(s) of heterodimerization modulate the bexarotene responses in intestinal cholesterol absorption is still open. Despite being expressed in the intestine, Peroxisome proliferator-activated receptor (PPAR)α is unlikely to be involved, because a previous study showed no effect of its activation on NPC1L1 gene expression.25 Conversely, PPARδ could be implicated because NPC1L1 is known to be reduced in the intestine on PPARδ activation.16 However, PPARδ mRNA levels are decreased in bexarotene-treated mice as compared with controls (data not shown), suggesting the existence of an alternative partner. Finally, the most attractive partner of RXR heterodimerization in the intestine is the liver-X receptor (LXR). Indeed, NPC1L1 is reduced after LXR activation.25 Surprisingly, expression of the ABCA1 gene, a positive LXR target gene, is decreased in the intestine of bexarotene-treated mice. In addition, expression of ABCG5 and ABCG8, other well-characterized LXR target genes,26,27 are also decreased in the intestine of bexarotene-treated mice (data not shown). Interestingly, treatment with bexarotene did increase aortic ABCA1 expression, but only modestly altered expression of genes implicated in the parietal inflammatory process, whereas LXR activation has been shown to exert anti-inflammatory effects.27 Thus it is unclear to what extent the LXR/RXR pathway mediates the effects of bexarotene and bexarotene certainly acts as a selective, tissue-specific, and gene-specific modulator of the LXR/RXR pathway.

Cholesterol efflux, the obligatory first step of reverse cholesterol transport, is a “bi-partner process.” On the one side are the acceptors of cholesterol, namely lipoproteins present in plasma and intercellular fluids, and on the other side the cells and their cholesterol transporters. Among them, ABCA1 and ABCG1 have been shown to specifically mediate cholesterol efflux from cells to lipid-poor apoa-I and to HDL, respectively.28,29 Treating mice with bexarotene did not significantly modify the capacity of plasma to accept cholesterol from cells ex vivo. By contrast, in vitro incubation of murine cholesterol-laden peritoneal macrophages with bexarotene resulted in a significant increase of lipid-free apoA-I-mediated cholesterol efflux. Moreover, bexarotene enhanced cholesterol efflux mediated by HDL, suggesting that both ABCA1 and ABCG1-mediated cholesterol efflux are facilitated on treatment of macrophages in vitro. Finally, the expression of these cholesterol transporters was enhanced in the aortic sinus, ie, the area enriched in macrophages, on in vivo treatment. Moreover, in vivo treatment decreased the number of lipid-loaded peritoneal macrophages, which correlates with less lipid-loading of the cell population present in the atherosclerotic lesions. Taken together, our present results suggest that macrophages in the aortic sinus of treated mice display enhanced capacity to efflux cholesterol, thus preventing foam cell formation and subsequent lesion development.

As the role of inflammation in atherosclerosis has been increasingly recognized at all stages of its pathogenesis, we measured the expression of genes encoding different cytokines and proteins implicated in the inflammatory process and previously shown to be regulated in vitro by retinoids (natural RXR ligands) or rexinoids (synthetic RXR ligands).30–34 Bexarotene treatment induced only modest variations in the genes encoding MCP-1 and macrophage colony-stimulating factor, two strong modulators of foam cell formation. In addition, in the subcutaneous dorsal pouch acute inflammatory model,35 bexarotene treatment did not exert any anti-inflammatory activity (data not shown) indicating that bexarotene has no marked effect in the inflammatory process in the vascular wall in vivo.

In our model, bexarotene-treatment induced a marked increase in triglyceridemia as it does in humans.1 Our results demonstrate that the biosynthesis of triglycerides could be affected by bexarotene treatment, because hepatic expression of SCD1 and FAS was increased. Interestingly, these genes are targets of the LXR/RXR heterodimer and LXR agonists also increase hepatic lipogenesis and plasma triglycerides.36 SCD1 protein activity appeared also increased by bexarotene treatment, as assessed by the higher desaturation index (C18:1/C18:0) of plasma cholesteryl esters in treated mice as compared with controls. In addition, the slight increase of hepatic expression of Angptl3, a protein identified as an inhibitor of lipoprotein–lipase,37 the enzyme responsible for catabolism of triglycerides in the vascular compartment, could also enhance the triglyceridemia. Although epidemiological and clinical studies demonstrated the association of elevated plasma triglyceride levels with increased risk of cardiovascular disease,38 it is interesting to note that increasing plasma triglyceride levels, as observed not only after bexarotene but also LXR agonist treatment, is not sufficient to aggravate atherosclerosis progression when associated with a decrease in non-HDL cholesterol and an improvement of lipid homeostasis in macrophages.

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Atheroscleroprotective Effect of Bexarotene in Mice

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Disclosures
None.

References
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Supplementary information published online

1) Materials and Methods

Lipid and lipoprotein analyses

Plasma concentrations of total cholesterol (TC) and triglycerides (TG) were measured using commercially available kits (Biomerieux, France). HDL cholesterol (HDL-C) concentrations were determined in plasma after precipitation of apolipoprotein (apo) B-containing lipoproteins by phosphotungstic acid/Mg (Boehringer Mannheim, Germany). Non-HDL cholesterol (N-HDL-C) was obtained by subtraction of HDL-C values from TC.

Lipoproteins were separated from 200 µl of pooled plasma by gel filtration chromatography using a Superose 6 HR 10/30 column (Pharmacia, Sweden). The gel was allowed to equilibrate with 10 mM phosphate–buffered saline (PBS) containing 0.01% (wt/vol) EDTA and 0.01% (wt/vol) sodium azide. Plasma was eluted with the buffer at room temperature at a flow rate of 0.2 ml.min⁻¹. The effluents were collected in 0.22-ml fractions. Cholesterol and triglyceride concentrations were determined in the eluted fractions as described above.

Plasma levels of mouse B were measured by immunonephelometric assays using specific polyclonal antibodies.
Fatty acid composition of plasma cholesteryl esters.

Lipids were extracted from plasma according to the Folch method (Folch et al.). The lipid extract was dried under nitrogen, dissolved in chloroform/methanol (2:1, v:v), and separated by thin layer chromatography using petroleum ether/ether/acetic acid (90:10:5, v:v:v). Cholesteryl esters were identified by simultaneous migration of standards. Cholesteryl esters were scrapped, hydrolysed and the resulting fatty acids were methylated by treatment with methalonic H$_2$SO$_4$ for 2 h at 70°C. The fatty acid methyl esters were extracted with heptane and analysed by gas chromatography on a Varian 3400 chromatograph (Palo Alto, CA) equipped with a flame ionisation detector and a capillary column. A gradient temperature from 160°C to 210°C at 1.5°C min$^{-1}$ and a N$_2$ pressure of 85 kPA were used. Identification of fatty acids was achieved using commercially available fatty acid methyl esters.

Reference:

Quantification of atherosclerotic lesions

After euthanasia, the heart was perfused from its apex with cold Krebs-Ringer buffer and fixed in a solution containing 4% paraformaldehyde in PBS, pH 7.4. The heart, containing the aortic origin, was carefully removed and fixed in paraformaldehyde overnight. The upper half of the heart was equilibrated in 30% sucrose, embedded in Tissue-Tek OCT compound and frozen in liquid nitrogen in combination with isopentane. Serial 10-µm-thick cryosections were obtained from the aortic arch to the ventricles and were used for detection of lipid deposition using Oil Red O and hematoxylin. Quantitative analysis of the atherosclerotic lesion area including the
positive Oil Red O staining area was performed every 5 sections, each 50 µm apart. Images were captured by use of a JVC 3-CCD video camera and analysed blindly by one observer using computer assisted-Quips Image analysis system from Leica (Leica Mikroskopic und System GmbH, Wetzlar, Germany). To avoid morphological heterogeneity between mice, the expression of average atherosclerotic lesion areas of the aortic root, from the appearance to the disappearance of the aortic valves, was normalized at ten equal sections.

**Cholesterol efflux experiments**

Cellular cholesterol efflux ability of plasma was determined using Fu5AH rat hepatoma cells following the procedure described by de la Llera Moya et al. Briefly, the cells were maintained in minimal essential medium containing 5% fetal calf serum. Fu5AH were plated on 24 multiwell plates (BD, Le Pont de Claix, France) using 1 mL per well. Two days after plating, cellular cholesterol was labelled during a 72 hour-incubation with $^{3}$H-cholesterol (Amersham, Orsay, France) (0.5 µCi/well). To allow equilibration of the label, the cells were washed and incubated for 24 hours in minimal essential medium with 0.5% wt/vol bovine serum albumin (BSA). Then the cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C for 4 hours with 2.5% (v:v) diluted plasma. At the end of the incubation, the medium was removed and centrifuged. The monolayer cells were washed 3 times with PBS and harvested with 0.5 mL of 1 mol/L NaOH. Radioactivity was then measured in both medium and cells, and percentage of cholesterol efflux calculated. Measurements were performed in triplicate. Thioglycollate-elicited mouse peritoneal macrophages plated at $10^5$ cells/ml were loaded with 50 µg/ml $[^3]$H acetylated low-density lipoproteins for 24 h in serum-free
DMEM containing 0.2 % (v:v) BSA. Loaded cells were then washed, activated for 24 hours with bexarotene (1µM) and efflux was initiated in medium containing 10 µg/ml of human apo A-I or 30 µg/ml of human HDL₃ isolated from plasma by ultracentrifugation at density 1.063<d<1.21. Twenty-four hours later, the medium was removed and centrifuged to remove cell debris. The cells were dissolved in 1 M NaOH. Radioactivity was then measured in both medium and cells, and percentage of cholesterol efflux calculated. Measurements were performed in triplicate.

Reference:

**Analysis of lipid-loading of peritoneal macrophages**

Mouse peritoneal macrophages (MPMs) were collected by peritoneal lavage with PBS from mice given a 3-mL intraperitoneal injection of 4% thioglycollate in water. The MPMs were pelleted, washed twice with serum-free RPMI (GIBCO), plated at a density of 1.5x10⁶ cells/35-mm dish, and allowed to adhere to dishes for 2 hours in RPMI. Then, plates were washed 3 times with PBS to remove nonadherent cells. Lipids were stained by Oil-Red-O according to the method of Lillie and Ashburn (1943). Cells were counterstained with hematoxylin.

**Intestinal cholesterol absorption**

Intestinal cholesterol absorption was measured by the dual fecal isotope ratio method, as described by Wang and Carey. Fecal lipids were extracted and
\[^{14}\text{C}]\text{cholesterol and }[^{3}\text{H}]\text{sitostanol levels determined. The ratio of the two}\]
\[^{14}\text{C}]\text{radiolabeled isotopes in the fecal extracts and the dosing mixture was used for}\]
calculation of percent cholesterol absorption:

\[
\text{% absorption} = \frac{[^{14}\text{C}]}{[^{3}\text{H}]} \text{dosing mixture} - \frac{[^{14}\text{C}]}{[^{3}\text{H}]} \text{feces} \times 100
\]
\[
\frac{[^{14}\text{C}]}{[^{3}\text{H}]} \text{dosing mixture}
\]

Reference:

**Real-time PCR-based quantitative gene expression analysis**

Total RNA was isolated from cells or tissues by the acid guanidium thiocyanate/phenol/chloroform method as previously described (Chomczynski 1987) and reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies, France). For quantitative PCR, reverse transcribed cDNA were quantified by real-time PCR on a MX 4000 apparatus (Stratagene), using specific oligonucleotide primers listed in Table 1. PCR amplification was performed in a volume of 25 µl containing 100 nM of each primer, 4 mM MgCl₂, the Brilliant Quantitative PCR Core Reagent mix as recommended by the manufacturer (Stratagene), and SYBR Green 0.33 X (Sigma-Aldrich, France). The conditions were 95°C for 10 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. All samples are run in duplicate. Results are expressed normalized to 28S RNA.

Reference:
Table I: Primers used for real-time PCR

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<th>Genes</th>
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<th>Reverse primers</th>
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<td>M-CSF</td>
<td>5'-AGC ATG GAC AGG CAG GGA C-3'</td>
<td>5'-CTG CGT GCC TTT ATG CCT TT-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-CCA GTT GCC TTC TTG GGA CTG-3'</td>
<td>5'-CAG GTC TGT TGG GAG TGG TAT CC-3'</td>
</tr>
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<td>IL12b</td>
<td>5'-GAC ATC ACC TGG ACC TCA GAC-3'</td>
<td>5'-AGT CTC GCC TCC TTT GTG GC-3'</td>
</tr>
<tr>
<td>TNFα</td>
<td>5'-AGC A CA GAA AGC ATG ATC CG-3'</td>
<td>5'-CCC GAA GTT CAG TAG ACA GAA GAG-3'</td>
</tr>
<tr>
<td>TF</td>
<td>5'-CAA GTG CTT CTC GAC CAG CAC C-3'</td>
<td>5'-TCC CCA TGA ACT GAG TTC TCT CG-3'</td>
</tr>
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<td>COX-2</td>
<td>5'-GGC CCT CTC TCC CGT AGC AG-3'</td>
<td>5'-AGA CCA GGC ACC AGA CCA AAG ACT-3'</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>5'-TCT CCA GCT TCT CTC AGG AAA TGC C-3'</td>
<td>5'-AAC CCA ATC CCC AAC TTG TGC AG-3'</td>
</tr>
<tr>
<td>ApoB</td>
<td>5'-GTG GCT TCT TCT GTC ACT GCT AAA G-3'</td>
<td>5'-GTC TGA TTT CCC CTC AAT GG-3'</td>
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<td>LDL-R</td>
<td>5'-CAG GCA GCA GGA AGC AGT TC-3'</td>
<td>5'-GGA GTC AGG AAT GCA TCG GC-3'</td>
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<tr>
<td>HMGCoA-S</td>
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<td>5'-TTT TGG CCG TAG GCT GCA TAG-3'</td>
</tr>
<tr>
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<td>5'-AGC CTT GCC TTG TAA GTT CTG TGG-3'</td>
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<tr>
<td>FAS</td>
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<td>5'-ATT GTG TGT GCC TGC TGG GG-3'</td>
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<tr>
<td>Angptl3</td>
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<td>5'-GCA GTC GGC AGG AAG GTC ATC TTG-3'</td>
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<td>NPC1L1</td>
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<td>5'-GCA AGG TGA TCA GGA GGT TGA-3'</td>
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<tr>
<td>CD13</td>
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<td>5'-AAG CCC TCG TTC AGC CAC AG-3'</td>
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<td>ABCA1</td>
<td>5'-ACA CCT GAC ACA CCA GCT AGA AGG C-3'</td>
<td>5'-TGA GTT CTT CCC ACA TGC CTT CC-3'</td>
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</table>
Statistical analysis

Values are reported as mean±SD. Statistical differences were determined by the non-parametric Mann-Whitney U-test. A value of p<0.05 was accepted as statistically significant.
Figure 7: Influence of bexarotene on the expression of genes encoding cytokines and inflammatory proteins in the aortic sinus of apoE2-KI mice.

Female apoE2-KI mice were treated with bexarotene (BEXA) (n=6) for 12 days and compared to control mice (CONT) (n=6). Data are expressed as percentages,
arbitrary values of 100% being attributed to the control group. Mean +/- SD. *p<0.05 versus controls.