

## Macrophage-mediated 15-Lipoxygenase Expression Protects against Atherosclerosis Development

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### Abstract

Oxidative modification of LDL increases its atherogenicity, and 15-lipoxygenase (15-LO) has been implicated in the process. To address this issue, we generated transgenic rabbits that expressed 15-LO in a macrophage-specific manner and studied their susceptibility to atherosclerosis development when they were fed a high-fat, high-cholesterol (HFHC) diet (Teklad 0533 rabbit diet 7009 with 10% corn oil and 0.25% cholesterol) for 13.5 wk. Transgenic and nontransgenic rabbits developed similar degrees of hypercholesterolemia and had similar levels of triglyceride, VLDL, LDL, and HDL. Quantitative morphometric analysis of the aortic atherosclerosis indicated that the transgenic animals ( $n = 19$ ) had significantly smaller lesion areas ( $9.8 \pm 6.5\%$ , mean  $\pm$  SD) than their littermate controls ( $n = 14$ ,  $17.8 \pm 15.0\%$ ) ( $P < 0.05$ ). In a subgroup ( $n = 9$ ) of transgenic rabbits that received the HFHC diet plus the antioxidant  $N',N'$ -diphenyl-phenylenediamine (1%), the extent of lesion involvement ( $9.8 \pm 7.5\%$ ) did not differ from the subgroup ( $n = 10$ ) that received the regular HFHC diet ( $9.7 \pm 5.9\%$ ). Since the results were unexpected, we repeated the experiments. Again, we found that the nontransgenic littermates ( $n = 12$ ) had more extensive lesions ( $11.6 \pm 10.6\%$ ) than the transgenic rabbits ( $n = 13$ ;  $9.5 \pm 7.8\%$ ), although the difference was not significant. In a third set of experiments, we crossed 15-LO transgenic rabbits with Watanabe heritable hyperlipidemic (WHHL) rabbits and found that the lesion area in the 15-LO transgenic/heterozygous WHHL rabbits ( $n = 14$ ) was only about one third ( $7.7 \pm 5.7\%$ ) that found in nontransgenic heterozygous WHHL littermate controls ( $n = 11$ ,  $20.7 \pm 19.4\%$ ) ( $P < 0.05$ ). These data suggest that overexpression of 15-LO in monocytes/macrophages protects against lipid deposition in the vessel wall during early atherogenesis in these rabbit models of atherosclerosis. (*J.*

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### Introduction

There is evidence that oxidative modification of LDL increases its atherogenicity (1, 2). LDL extracted from human and rabbit atherosclerotic lesions has properties very similar to oxidatively modified LDL (3). Two oxidation products, malondialdehyde and 4-hydroxynonenal-conjugated LDL, have been demonstrated in rabbit atherosclerotic lesions by immunohistochemical techniques (4, 5). Dietary supplementation of the antioxidants probucol, butylated hydroxytoluene, or  $N',N'$ -diphenyl-phenylenediamine to Watanabe heritable hyperlipidemic (WHHL)<sup>1</sup> rabbits or normal rabbits fed a high cholesterol diet inhibited the development of aortic atherosclerotic lesions (6–9). Autoantibodies against oxidized LDL have been detected in humans and, in one study, their titers show a positive correlation with the progression of carotid atherosclerosis (10).

The mechanisms responsible for the oxidation of LDL in vivo are not known. However, there is substantial evidence indicating that 15-lipoxygenase (15-LO) plays a role (reviewed in references 1, 2); in fact, many pharmaceutical companies have been screening for 15-LO inhibitors as potential antiatherosclerosis agents, based on the widely held belief that 15-LO is a proatherogenic molecule.

Although it is clear that 15-LO may represent one mechanism for the oxidation of LDL, the relative contribution of 15-LO to LDL oxidation or to atherosclerosis development is unknown. Furthermore, the presumed role of 15-LO in atherogenesis has never been studied in vivo. To this end, we have generated transgenic rabbits that express 15-LO in a macrophage-specific manner. Two types of 15-LO transgenic rabbits were studied, those with a wild-type New Zealand white genetic background and those with a heterozygous WHHL background. We examined the degree of aortic atherosclerosis in response to a high fat, high cholesterol diet feeding. To our surprise, we found that, compared with nontransgenic littermate controls, the 15-LO transgenic rabbits had a reduction in diet-induced aortic atherosclerosis as deter-

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1. Abbreviations used in this paper: 13-HODE, 13-hydroxy-9,11(Z,E)-octadecadienoic acid; 15-LO, 15-lipoxygenase; DPPD,  $N',N'$ -diphenyl-1,4-phenylenediamine; HFHC, high fat, high cholesterol; WHHL, Watanabe heritable hyperlipidemic.

mined by quantitative morphometry. Thus, contrary to popular belief, in these animals, the net overall effect of 15-LO appears to be antiatherogenic in nature.

## Methods

**Transgenic animal experiments.** All animal experiments were performed in accordance with Baylor College of Medicine institutional guidelines. Transgenic rabbits were produced by a protocol described previously (11). The transgene contains a 2.7-kb human 15-LO cDNA (12), a generous gift from Dr. Elliott Sigal (Syntex, Palo Alto, CA). A portion of the human apolipoprotein A-I gene containing the 5'-untranslated region and the first intron was ligated 5' to the 15-LO cDNA. This intron was included to enhance transgene expression. This hybrid A-I/15-LO cDNA was inserted into a chicken lysozyme genomic DNA containing 11.5-kb 5'- and 5.5-kb 3'-flanking sequences that include the A elements on both sides of the structural gene (13). The donor and recipient rabbits were both New Zealand white rabbits. The microinjection and subsequent screening were as described (11). Positive animals were bred with wildtype New Zealand white rabbits, and transmission of the transgene into the progeny was determined by Southern blot analysis of ear DNA. Some of the 15-LO transgenic rabbits were crossbred with WHHL rabbits that had been derived from English half-lop rabbits (14). Transgenic rabbits and their nontransgenic littermates were fed Diet A, a high fat, high cholesterol diet (Teklad 0533 rabbit diet 7009 containing 10% corn oil and 0.25% cholesterol, Teklad Premier Laboratory Diets, Madison, WI). In Experiment 1, a subgroup of transgenic rabbits was fed Diet B, which is Diet A plus 1% *N,N'*-diphenyl-1,4-phenylenediamine (DPPD). The DPPD was included to test the hypothesis that it might inhibit the LDL-oxidizing effects of 15-LO. Each animal received 125 g of the chow per day for 13.5 wk. At different times, blood was collected in EDTA tubes from the ear vein of anesthetized rabbits. Total cholesterol and triglyceride were determined by enzymatic kits (Sigma Chemical Co., St. Louis, MO). Lipoprotein fractions of different densities were isolated by sequential ultracentrifugal flotation (15) from 2 ml of plasma. The density of plasma was sequentially adjusted by potassium bromide to isolate VLDL (< 1.006 g/ml), LDL (1.006–1.063 g/ml) and HDL (1.063–1.21 g/ml) by ultracentrifugation of the samples at 40,000 rpm in a 70.1 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) at 10°C for 20, 24, and 48 h, respectively. The cholesterol and triglyceride content of each fraction was measured by enzymatic kits (Sigma Chemical Co.). The diet feeding, plasma lipid/lipoprotein measurements, and isolation of rabbit aortas were performed at Baylor College of Medicine; the samples were coded and sent to The Ohio State University for the morphometric analyses by investigators (E. Herderick and J.F. Cornhill) who were blinded to the identity of the treatment groups.

**15-Lipoxygenase assay.** 15-LO activity was determined as described previously (11). Briefly, peripheral monocytes were prepared from transgenic and nontransgenic rabbits by Ficoll-Hypaque density gradient centrifugation and adherence to plastic dishes (16). After scraping from the dishes, the cells were resuspended in PBS ( $2-5 \times 10^6$  cells/ml) and exogenous arachidonic acid was added to a final concentration of 160  $\mu$ M. Incubation was for 15 min at 37°C. The reaction was stopped by addition of equal volumes of a mixture of isopropanol/chloroform (1:1 by vol) and the lipids were extracted. The extracts were dried under vacuum, the lipids were reconstituted in 100  $\mu$ l of methanol, and the aliquots were injected to HPLC analysis for quantitation of 15S-hydroxy-5,8,11,14(Z,Z,Z,E)-eicosatetraenoic acid (15S-HETE) and 12S-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12S-HETE) (11).

**Quantitative morphometry of rabbit aortic atherosclerosis.** The aortas were fixed in 10% formalin and stained with Sudan IV (17). The extent of sudanophilia was determined using image analysis techniques employed previously to study the topographic distribution of sudanophilic lesions in minipigs (18). These methods include image

capture, transformation of the data to a standard template, image segmentation, and finally computation of probability-of-occurrence maps. The images were scanned on an Eikonix 7899 digital scanner at a resolution of  $2,048 \times 256$  pixels in 24-bit full color. A binary image for each aorta was created by classifying each pixel of the scanned image as Sudan positive or negative based on a supervised bayesian color classification algorithm. Each binary image was transformed to a standard template to remove anatomical variations among individuals. The standard template was created by calculating the average location of anatomical landmarks for all individuals in the study. The stored transformed binary images were then used to calculate probability-of-occurrence maps by computing the probability of finding a Sudan-positive value at each pixel in the standard template. These maps can be displayed as incidence interval isopleths (0–10%, 10–20%, etc.). In addition, for each individual aorta, the percentage of Sudan-positive area was computed by dividing the number of positive pixels by the total number of pixels in the image. Using the Statistical Analysis System (SAS Institute, Cary, NC), a one-way ANOVA was performed to test the hypothesis that there was no difference in the mean percent-positive values between groups. The results are presented later in Table II.

**HPLC analysis of linoleic acid oxidation.** Cell preparation, incubation, and analyses were carried out as described for the 15-lipoxygenase assay except that linoleic acid instead of arachidonic acid was used as substrate. HPLC was carried out on a Shimadzu instrument (Shimadzu Scientific Instruments, Inc., Columbia, MD) coupled with a diode array detector (1040 A; Hewlett-Packard Co., Palo Alto, CA). Reverse-phase HPLC was performed on a Nucleosil C-18 column (Macherey/Nagel, Bad Dürren, Germany; KS-system,  $250 \times 4$  mm, 5  $\mu$ m particle size). A solvent system of methanol, water, and acetic acid (85, 15, and 0.1 by vol, respectively) and a flow rate of 1 ml/min was used. The fractions containing the oxygenated polyenoic fatty acids were collected, the solvent was evaporated, the residue was reconstituted in a mixture of *n*-hexane, 2-propanol, acetic acid (100, 2, and 0.1 by vol, respectively) and injected to straight-phase HPLC SIL column ( $250 \times 4.6$  mm, 5  $\mu$ m particle size; DuPont-Merck Pharmaceutical Co., Wilmington, DE) with a solvent system of *n*-hexane, 2-propanol, and acetic acid (100, 2, and 0.1 by vol, respectively) and a flow rate of 1 ml/min. Fractions containing 13-hydroxy-9Z,11E-octadecadienoic acid were collected, concentrated under vacuum, and enantiomer composition determined by chiral-phase HPLC that was carried out on a Chiralcel OD column ( $250 \times 4.6$  mm, 5  $\mu$ m particle size; Diacel Chemical Industries, distributed by J.T. Baker, Deventer, The Netherlands) with a solvent system of hexane, 2-propanol, and acetic acid (100, 5, and 0.1 by vol, respectively) and a flow rate of 1 ml/min.

## Results

**Transgenic rabbits expressing human 15-LO.** Previous studies on the role of 15-LO in atherosclerosis have been confined to *in vitro* analysis of 15-LO action or to correlative observations on 15-LO expression and atherogenesis in animals or humans. To determine more directly the role of 15-LO in atherosclerosis *in vivo*, we have produced transgenic rabbits (11) that overexpress human 15-LO (12). By Northern and Western blot analysis, these animals express 15-LO in their monocyte-derived macrophages, but not in any of the other tissues (liver, uterus, lung, adrenal, brain, kidney, heart, muscle, small intestine, and ovary) tested (11). The level of expression was high. By incubating the monocyte-derived macrophages (prepared as described in Methods) with exogenous arachidonic acid substrate, transgenic macrophages produced  $5.5 \pm 5.0$   $\mu$ g HETE compared with  $1.1 \pm 1.5$   $\mu$ g HETE per  $10^7$  cells in controls,  $P < 0.01$ . This level of 15-LO activity was comparable to that of interleukin-4-treated human monocytes (19).

Table I. Cholesterol Concentration in Total Plasma and Lipoprotein Fractions

	Number	Cholesterol (mg/dl)±SD			
		VLDL	LDL	HDL	Total plasma
Experiment 1: New Zealand white rabbits					
Prediet					
Transgenic	19	0.71±0.85	24.68±14.44	20.79±7.31	44.68±16.79
Nontransgenic	14	1.52±1.64	17.45±10.39	26.20±5.55	47.69±16.43
Week 12					
Transgenic	19	224.01±63.14	244.93±60.86	32.65±12.63	865.92±247.86
Nontransgenic	14	225.11±35.48	222.26±57.32	35.34±13.57	931.16±184.13
Experiment 2: New Zealand white rabbits					
Prediet					
Transgenic	9	3.58±2.94	18.16±10.32	29.98±6.57	48.91±12.12
Nontransgenic	9	3.46±1.97	30.67±17.99	32.89±7.66	58.67±13.88
Week 12					
Transgenic	13	234.22±78.21	231.34±83.60	61.25±19.09	704.00±376.64
Nontransgenic	12	286.04±38.27	301.34±100.85	70.69±20.49	810.60±298.98
Experiment 3: WHHL rabbits					
Prediet					
Transgenic	5	3.27±2.08	31.64±25.94	25.09±8.55	49.56±21.50
Nontransgenic	3	4.45±1.91	10.64±5.55	26.48±14.86	41.31±19.63
Week 12					
Transgenic	14	281.38±75.94	341.64±123.07	71.20±47.95	726.77±260.35
Nontransgenic	11	327.09±23.29	399.54±107.20	55.07±26.68	875.83±189.66

Lipids values determined at 4 and 8 wk also showed no difference between transgenic and nontransgenic animals (data not shown).

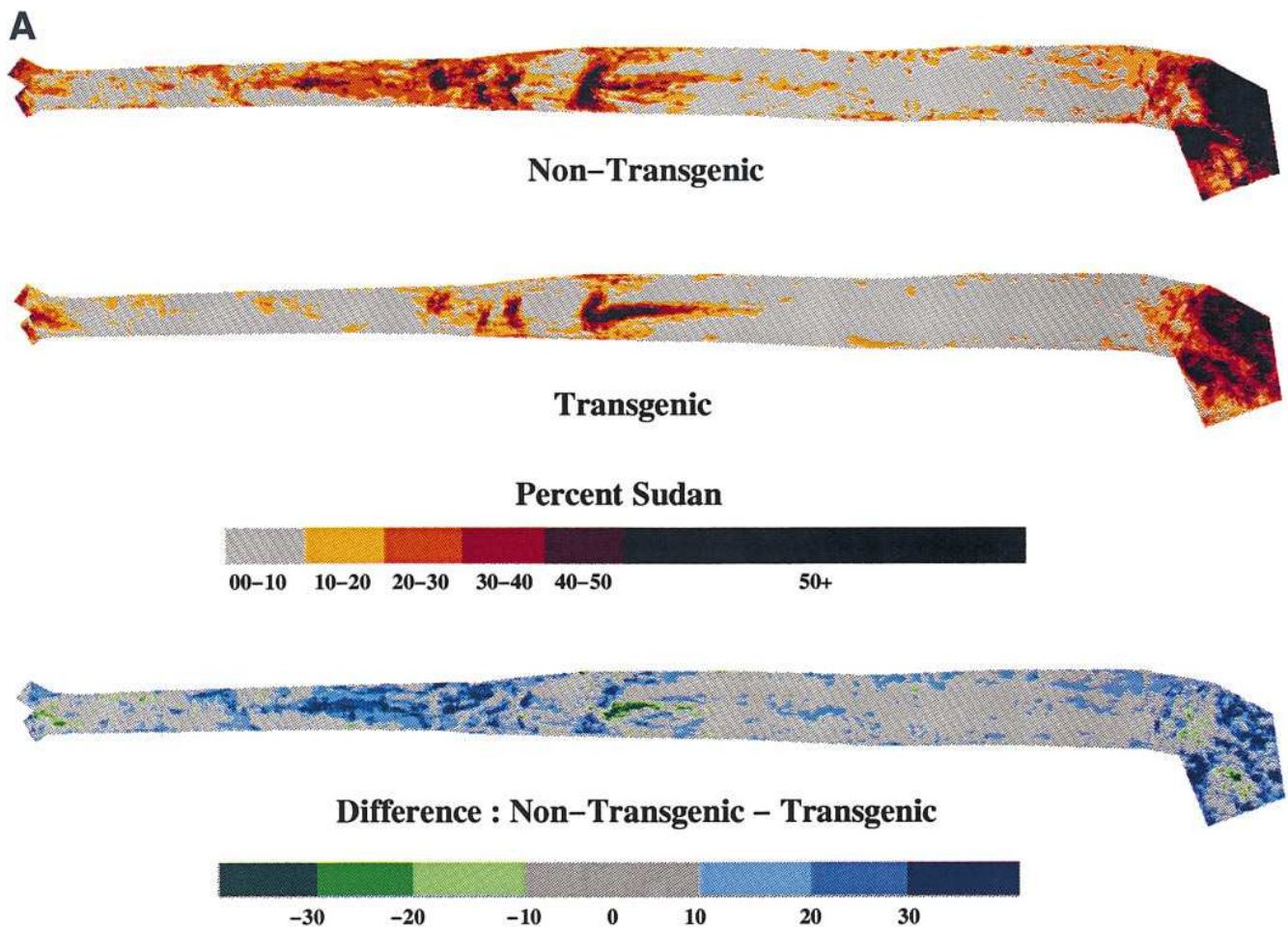
High fat, high cholesterol diet feeding in 15-LO transgenic rabbits with a wild-type New Zealand white background. We fed the transgenic rabbits and their littermate controls a high fat, high cholesterol (HFHC) diet containing 10% corn oil and 0.25% cholesterol without (Diet A) or with (Diet B) 1% DPPD for 13.5 wk. Since Diet A- and Diet B-transgenic animals developed the same degree of hyperlipidemia, their lipid values are considered together in Table I. The transgenic and nontransgenic animals developed comparable hypercholesterolemia (Table I, Experiment 1). The plasma triglyceride remained un-

changed at 50.77±10.49 mg/dl (transgenic) and 55.83±13.37 mg/dl (nontransgenic) before diet treatment and 100.12±39.26 mg/dl (transgenic) and 112.88±45.37 mg/dl (nontransgenic) at 12 wk after diet treatment. The plasma VLDL, LDL, and HDL cholesterol concentrations were also comparable in the two groups of animals (Table I, Experiment 1).

After the animals were on the HFHC diet for 13.5 wk, they were killed and the extent of aortic surface involvement with atherosclerosis was determined by quantitative morphometry (18). When stained with Sudan IV, the thoracic aortas of both

Table II. Aortic Atherosclerotic Lesion Involvement in 15-Lipoxygenase Transgenic Rabbits and Nontransgenic Littermate Controls

	Number	Lesion involvement percent±SD	P value
Wild-type New Zealand white background			
Experiment 1			
Nontransgenic diet A	14	17.8±15.0	
Transgenic diet A	10	9.7±5.9	0.12 (vs nontransgenic)
Transgenic diet B	9	9.8±7.5	0.15 (vs nontransgenic)
Transgenic diet A+B	19	9.8±6.5	0.04 (vs nontransgenic)
Experiment 2 (diet A)			
Nontransgenic	12	11.6±10.6	
Transgenic	13	9.5±7.8	0.57
Heterozygous WHHL genetic background			
Experiment 3 (diet A)			
WHHL-nontransgenic	11	20.7±19.4	
WHHL-transgenic	14	7.7±5.7	0.03



**Figure 1.** Probability of occurrence of sudanophilia in the rabbit aorta. The method for the quantitative morphometry is as described in Methods. The maps for the nontransgenic and transgenic groups are displayed in banded incidence isopleths. The difference between the transgenic and nontransgenic groups is displayed at the bottom. The values in this difference map are computed by calculating at each pixel the difference between the nontransgenic and transgenic groups. Blue values indicate that the incidence in the nontransgenic group is greater than the corresponding pixel in the transgenic group. Green values indicate the incidence is greater in the transgenic group. (A) Experiment 1: New Zealand white rabbits with ( $n = 19$ ) and without ( $n = 14$ ) the integrated human 15-LO transgene were studied. (B) Experiment 2: New Zealand white rabbits that were the progeny of those in Experiment 1 were used in this experiment a year later, after a rabbit breeding program. Number of animals were: transgenic, 13; nontransgenic littermates, 12. (C) Experiment 3: heterozygous WHHL rabbits with ( $n = 14$ ) and without ( $n = 11$ ) the 15-LO transgene obtained by crossbreeding were used in this experiment.

transgenic and nontransgenic rabbits showed heavy lesion involvement. The abdominal aorta, in contrast, was generally not as extensively covered by the lesions. It was, however, much more extensively involved in the nontransgenic animals compared with the transgenic group. The areas covered by Sudan IV-positive atherosclerotic lesions are  $17.8 \pm 15.0\%$  for the nontransgenic group,  $9.7 \pm 5.9\%$  for the transgenic group fed Diet A, and  $9.8 \pm 7.5\%$  for the transgenic group fed Diet B (Diet A + 1% DPPD). When the total combined transgenic group lesion size was compared with that of the nontransgenic animals, the difference was highly significant ( $P = 0.04$ ) (Table II). The histology of the atherosclerotic lesions in the transgenic and nontransgenic groups of animals is very similar (data not shown). All transgenic animals were combined for the probability mapping analysis. The probability-of-occurrence maps, Experiment 1 (Fig. 1 A), revealed that in both transgenic and nontransgenic animals, the highest probability of lesion formation occurred in the aortic arch area, especially on

the side of the greater curvature. In the abdominal aorta, in the nontransgenic group, the area just distal to the mesenteric ostium showed a 20–40% probability of involvement, compared with only a < 10–20% probability of a similar area in the transgenic group. The difference map at the bottom of Fig. 1 A confirms that the major difference in the probability of atherosclerotic lesion involvement occurs in the abdominal aorta (different shades of blue in the figure). However, the probability of occurrence is also greater in the aortic arch of the nontransgenic animals. Throughout the entire surface area of the aorta, the difference maps almost uniformly display areas of equal or greater probability for the nontransgenic animals compared with the transgenic animals. Only in a few scattered areas is the converse (i.e., higher probability in the transgenic group, green areas in the difference map) true.

It is evident in Table II that the presence of the human 15-LO transgene was associated with an  $\sim 47\%$  reduction in atherosclerosis involvement of the aorta. The inclusion of the antiox-

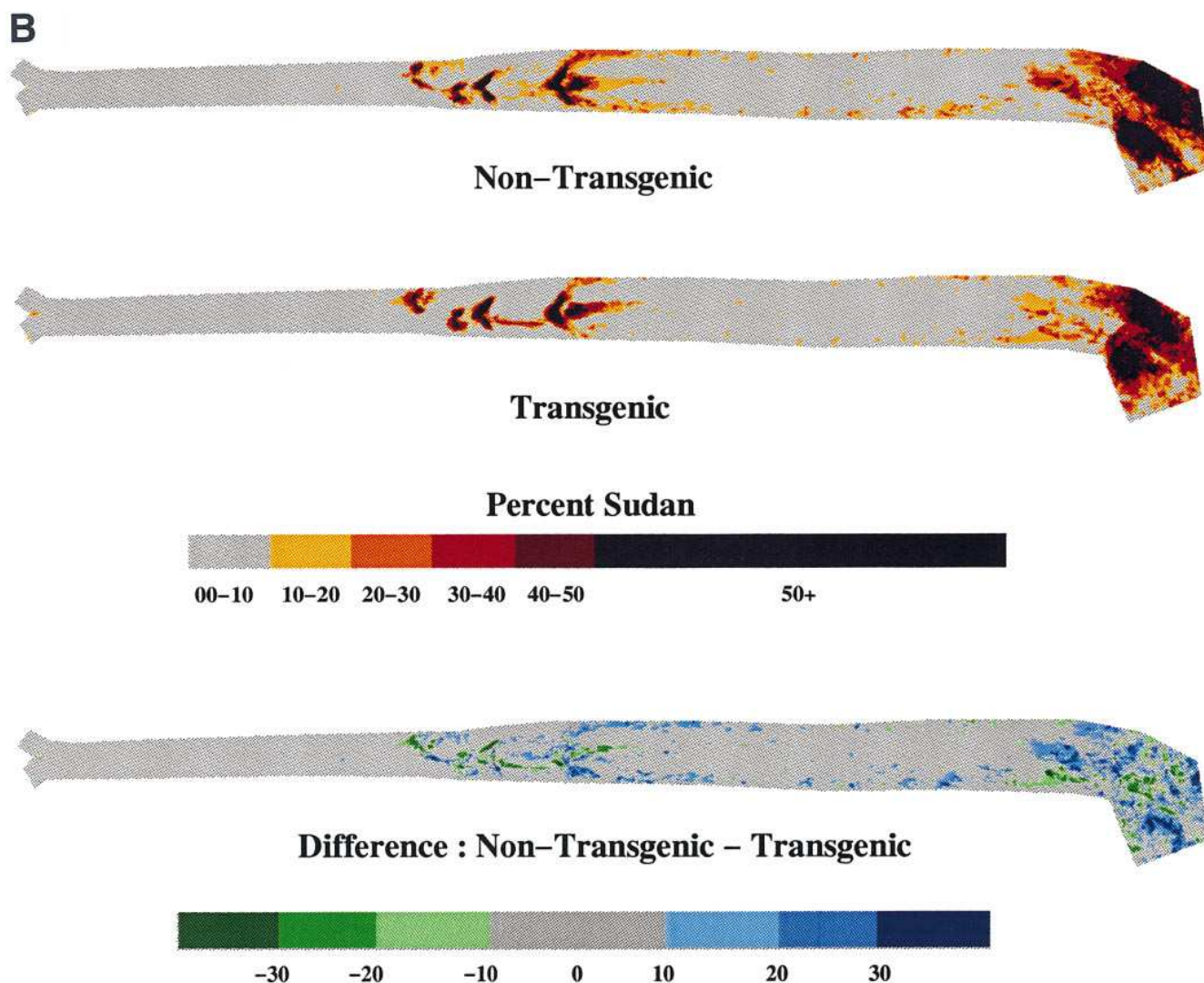


Figure 1. Continued.

identant DPPD in the diet did not change the degree of involvement in the transgenic group. Thus, in this experiment, macrophage-specific 15-LO overexpression was protective against diet-induced atherosclerosis in New Zealand white rabbits.

Because the results were totally unexpected, we decided to repeat the experiment using another group of 15-LO transgenic rabbits and their littermate controls obtained by cross-breeding. Again, in response to the HFHC Diet A feeding, there was no difference in the total plasma or lipoprotein cholesterol between the two groups of animals (Table I, Experiment 2). The plasma triglyceride was  $58.57 \pm 21.84$  mg/dl (transgenic) and  $54.85 \pm 15.71$  mg/dl (nontransgenic) before, and  $111.40 \pm 80.54$  mg/dl (transgenic) and  $93.06 \pm 43.15$  mg/dl (nontransgenic) at 12 wk of diet treatment. At the end of 13.5 wk, morphometric analysis of the rabbit aortas indicates that there was a statistically insignificant reduction in the extent of aortic atherosclerosis in the transgenic group ( $9.5 \pm 7.8\%$ ) compared with the nontransgenic group ( $11.6 \pm 10.6\%$ ) (Table II). The probability maps, Experiment 2 (Fig. 1 B), revealed that the aortic arch showed the highest probability of lesion involvement. The difference map at the bottom of Fig. 1 B confirms that there were only minor differences in probability in

lesion involvement in the aortic arch and around the celiac and mesenteric orifices. Blue areas slightly predominate over green areas in this map, consistent with a slightly higher probability of lesion involvement in the nontransgenic group.

*High fat, high cholesterol diet feeding in 15-LO transgenic rabbits with a heterozygous WHHL genetic background.* To further define the role of 15-LO in atherosclerosis development in an animal model of familial hypercholesterolemia, we intercrossed the 15-LO transgenic rabbits with WHHL rabbits. Two groups of animals consisting of littermate heterozygous WHHL rabbits, with and without the 15-LO transgene, were fed the same HFHC Diet A for 13.5 wk. Again, there was no difference in their total plasma cholesterol lipids in response to the diet feeding. The plasma VLDL, LDL, and HDL cholesterol were similar in the transgenic and nontransgenic WHHL rabbits (Table I, Experiment 3) throughout the treatment period. The plasma triglyceride was  $55.54 \pm 12.73$  mg/dl (WHHL transgenic) and  $58.94 \pm 7.94$  mg/dl (WHHL nontransgenic) before, and  $41.43 \pm 29.35$  mg/dl (WHHL transgenic) and  $40.21 \pm 25.04$  mg/dl (WHHL nontransgenic) at 12 wk after diet treatment. (The data in the WHHL rabbits should not be compared with those from Experiments 1 and 2 because the experiments were performed at different times and the WHHL rab-

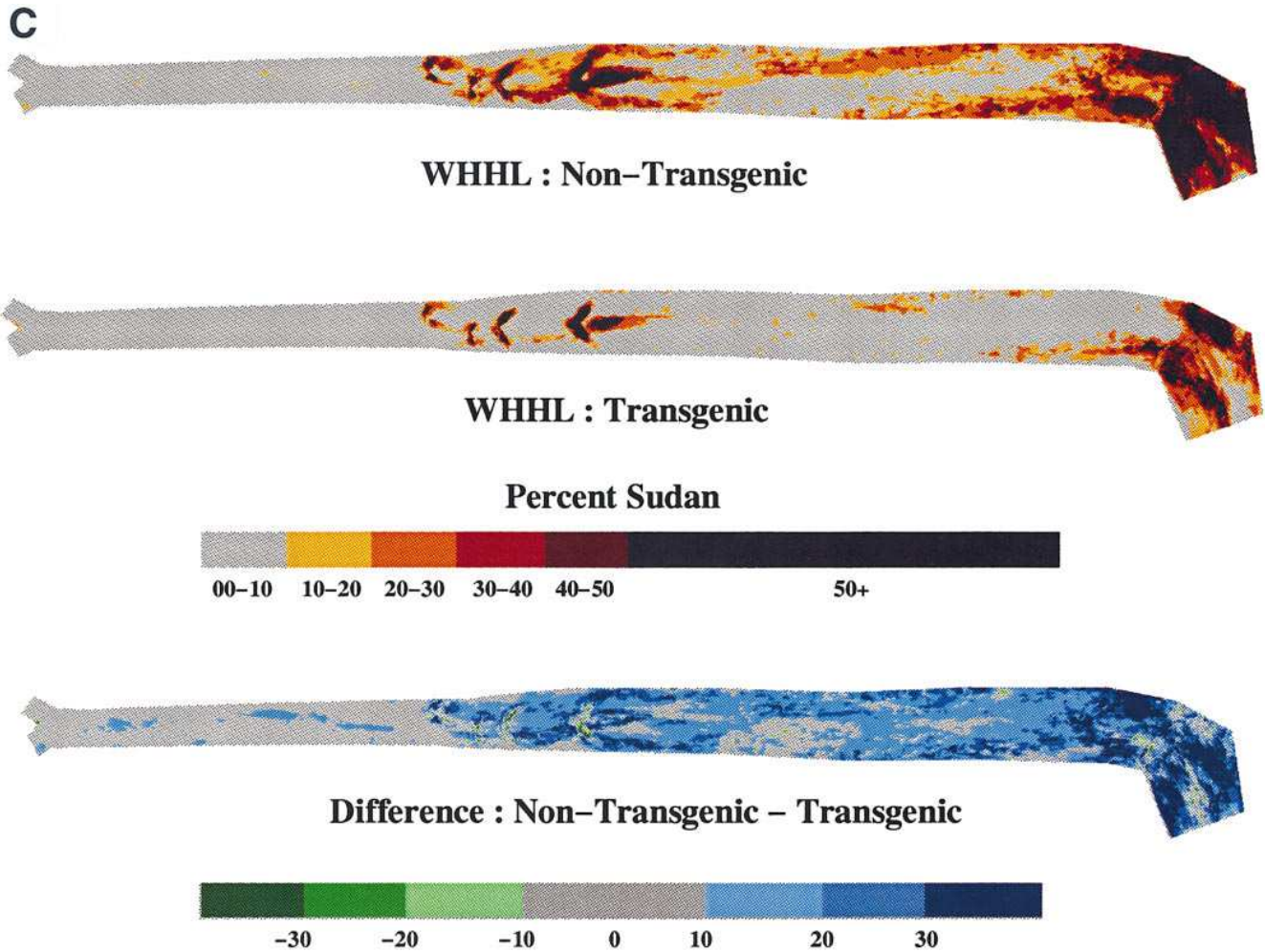


Figure 1. Continued.

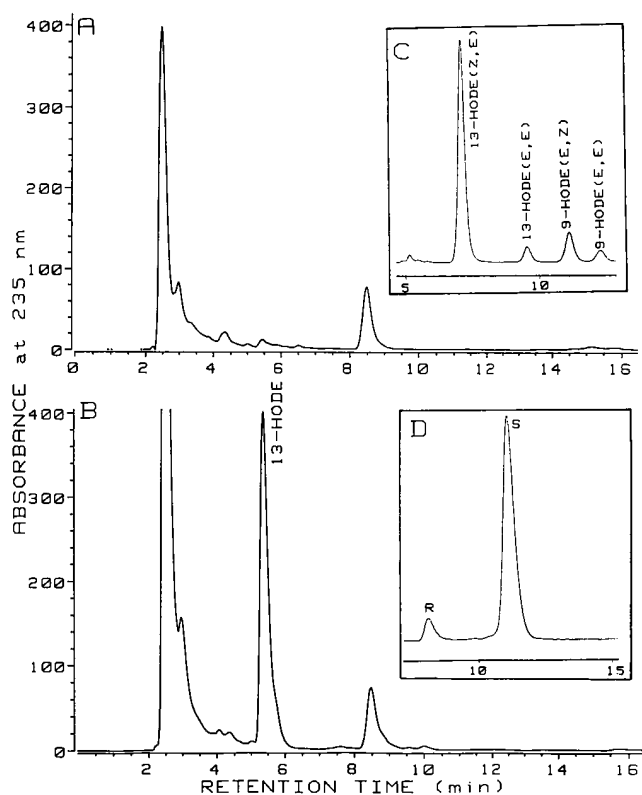
bits were derived from a different strain [English half-lop rabbits, reference 14].) When the size of the atherosclerotic lesion in the aortas of heterozygous WHHL rabbits with or without an integrated 15-LO transgene was compared, it was clear that the presence of the 15-LO transgene protected against the development of atherosclerosis in these animals (Table II and Experiment 3, Fig. 1 C). Although the general distribution of lesions is similar in these WHHL rabbits compared with New Zealand white rabbits, the probability-of-occurrence maps reveal some interesting differences (Fig. 1 C). As in wild-type New Zealand white rabbits, the aortic arch of the nontransgenic heterozygous WHHL rabbit displays the highest probability of lesion involvement. This is followed by the areas around the celiac, mesenteric, and renal ostia. In these nontransgenic WHHL rabbits, there are areas of 30–40% lesion probability in parts of the thoracic descending aorta. All these areas were much less extensively involved in the 15-LO transgenic WHHL rabbit aortas. The difference map (Fig. 1 C, bottom) shows blue and dark blue areas throughout the aortic arch, the entire thoracic aorta and the proximal abdominal aorta, in which there are substantially higher probabilities of lesion involvement in the WHHL rabbits without the 15-LO transgene compared with those with the transgene. Direct quantitation of sudanophilic areas indicates that the nontransgenic WHHL aorta displayed a  $20.7 \pm 19.4\%$  involvement,

which was reduced to about one third, or  $7.7 \pm 5.7\%$  involvement ( $P = 0.03$ ), in the presence of the 15-LO transgene (Table II). The results in Experiment 3 are thus quite consistent with those in Experiment 1. Although the data in Experiment 2 showed a statistically insignificant protection by 15-LO, the minor difference between transgenic and control animals was in the same direction as in the other two experiments. Thus, the combined results from all three experiments are consistent with 15-LO having a protective effect on atherosclerosis development.

*Transgenic, but not nontransgenic, macrophages convert linoleic acid to 13-hydroxy-9,11(Z,E)-octadienoic acid.* When linoleic acid was incubated with rabbit monocyte-derived macrophages in vitro, only macrophages from 15-LO transgenic rabbits, and not those from nontransgenic littermates, produced a 13-hydroxy-9,11(Z,E)-octadienoic acid (13-HODE) product (Fig. 2).

## Discussion

We initiated the 15-LO transgenic rabbit experiments because of our belief that 15-LO was a proatherogenic molecule that was responsible, at least partly, for the oxidative modification of LDL. We used rabbits for these experiments because (a) the



**Figure 2.** HPLC analysis of linoleic acid oxygenation products formed by peripheral monocyte-derived macrophages prepared from normal and transgenic rabbits. (A) Reverse phase-HPLC of products formed by monocyte-derived macrophages of nontransgenic rabbits. (B) Reverse phase-HPLC of products formed by monocyte-derived macrophages of nontransgenic rabbits; note the appearance of the 13-HODE peak absent in A. (C) Straight phase-HPLC of products formed by monocyte-derived macrophages of transgenic rabbits. (D) Chiral phase-HPLC of products formed by monocyte-derived macrophages of transgenic rabbits.

size of these animals allowed us to repeatedly monitor the plasma lipid and lipoprotein response; (b) many of the conclusions on the atherogenic potential of 15-LO and oxidized lipoproteins were based on experiments performed in rabbits (3, 4, 6–9, 20, 21); (c) two of us (J.F. Cornhill and E. Herderick) have developed a reliable method for quantitative morphometric analysis of rabbit aortic atherosclerotic lesions (18); and (d) the mouse, another popular animal model of atherosclerosis, has 12-LO but no 15-LO and is thus not as suitable a model for study. Three sets of experiments were performed consecutively over a 3-yr period because of the time needed for cross-breeding, diet feeding, and data analysis. The results of these experiments (Fig. 1 and Table II) indicate that the presence of the 15-LO transgene was associated with a reduction in the extent of diet-induced aortic atherosclerosis in the presence of similar plasma lipid and lipoprotein levels (Table I). We believe that the results in the WHHL rabbits (Experiment 3) are the most revealing. Here, the highly significant reduction in atherosclerotic lesion involvement occurs in an animal model of familial hypercholesterolemia.

Although unexpected, we do not believe that the observed protective effect of 15-LO transgene expression in any way negates the hypothesis that oxidized LDL is much more athero-

genic than unoxidized LDL. Although there is still some controversy regarding the exact mechanisms involved in LDL oxidation (22, 23), there is general agreement that oxidized LDL is a "harmful" molecule with regard to foam cell formation and atherosclerosis development. The explanation for the antiatherogenic effect of the 15-LO transgene must lie elsewhere.

One possibility for the experimental observations presented in this study is that 15-LO is totally uninvolved in the oxidative modification of LDL. We do not believe that this is a likely explanation because of the overwhelming evidence implicating the enzyme in the oxidative process. The different lines of evidence can be summarized as follows: (a) purified 15-LO can oxidatively modify LDL in cell-free systems (24, 25); (b) exposure of LDL to murine fibroblasts expressing high levels of human 15-LO results in enhanced levels of lipoperoxides (26); (c) oxidation of LDL by cultured endothelial cells and macrophages is inhibited in the presence of 15-LO inhibitors (24, 27); (d) 15-LO mRNA and protein (20, 28) as well as enzymatic activity (29) are expressed in macrophage-rich areas of human and rabbit atherosclerotic tissues; (e) somatic gene transfer of 15-LO into rabbit iliac arteries leads to the appearance of oxidized LDL-specific epitopes (21); and (f) increased amounts of stereospecific products of 15-LO-mediated oxidation products have been demonstrated in early atherosclerotic lesions in rabbits fed a high fat, high cholesterol diet (29). Despite the strong evidence implicating 15-LO in LDL oxidation, the quantitative role of this enzyme in the process is unknown. The design of the experiments on 15-LO transgenic rabbits does not allow us to directly address the issue of whether 15-LO mediates LDL oxidation. In the face of the lines of evidence for the involvement of 15-LO in the oxidative process summarized above, we believe that other explanations for our experimental results must be sought.

The explanation we favor is that 15-LO has multiple actions *in vivo*, some of which, like LDL oxidation, are proatherogenic, whereas others may be antiatherogenic. The potential antiatherogenic actions of 15-LO are unknown, but we can speculate on some effects of the enzyme that may play a role. 15-LO is known to convert linoleic acid, the predominant essential fatty acid in the vessel wall, to 13-HODE (30). 13-HODE is a platelet chemorepellant factor produced by endothelial cells (31, 32) that inhibits platelet adhesion to these cells (31–33). 13-HODE also stimulates prostacyclin production by endothelial cells (34) and decreases thromboxane production in platelets (35), effects that are antiatherogenic and anti-thrombogenic. We have tested the capacity of transgenic and control rabbit macrophages to produce 13-HODE from linoleic acid *in vitro* and found that this compound was the major oxygenation product produced by transgenic macrophages, but was not detectable with the nontransgenic macrophages (Fig. 2). It has been proposed that the induction of 15-LO in the atherosclerotic lesion may represent a protective response (36). Thus, there could be other antiatherogenic effects of 15-LO that will be subjects of future investigations. In the meantime, it is important to recognize that, in the transgenic and control rabbits, the net overall effect of macrophage-mediated 15-LO expression is not proatherogenic and may well be protective. These observations have implications for the pathogenesis of atherosclerosis; they also suggest that the premise that specific 15-LO inhibitors are effective antiatherosclerotic pharmaceuticals needs reconsideration.

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