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Human apolipoprotein A-II is a pro-atherogenic molecule when it is expressed in transgenic mice at a level similar to that in humans: evidence of a potentially relevant species-specific interaction with diet

Joan Carles Escolà-Gil,[*,†] Àfrica Marzal-Casacuberta,[*,†,§] Josep Julve-Gil,[*,†] Brian Y. Ishida,[**] Jordi Ordóñez-Llanos,[*,§] Lawrence Chan,[††] Francesc González-Sastre,[*,§] and Francisco Blanco-Vaca[1,*,†]

Servei de Bioquímica,* Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau,† Barcelona, Spain; Departament de Bioquímica i Biologia Molecular,§ Universitat Autònoma de Barcelona, Barcelona, Spain; Department of Medicine,** University of California-San Francisco, San Francisco, CA; and Departments of Cell Biology and Medicine,†† Baylor College of Medicine, Houston, TX

Abstract We report on the effect of human apolipoprotein (apo) A-II transgene expression on atherosclerosis susceptibility in two transgenic lines (25.3 and 11.1) whose plasma human apoA-II concentrations (~23 and 96 mg/dl, respectively) span the normal range in humans. After 9 months of an atherogenic diet, 25.3 and 11.1 transgenic mice developed aortic atherosclerotic lesions that were ~1.7- and 7-fold, respectively, more extensive than those of non-transgenic control mice. However, there was no difference in the area of atherosclerosis of transgenic and control mice when fed a regular chow diet. This contrasts with the findings in murine apoA-II transgenic mice and provides evidence of a species-specific characteristic that could be of relevance with respect to the high fat intake diets common in most industrialized countries. A possible mechanism of the pro-atherogenic action of human apoA-II could be the inhibition of reverse cholesterol transport and, in support of this, we observed an impairment of apoA-I-HDL particle interconversion in the plasma of 11.1 transgenic mice caused, at least in part, by a marked decrease in the endogenous lecithin:cholesterol acyltransferase activity.—**Escolà-Gil, J. C., À. Marzal-Casacuberta, J. Julve-Gil, B. Y. Ishida, J. Ordóñez-Llanos, L. Chan, F. González-Sastre, and F. Blanco-Vaca.** Human apolipoprotein A-II is a pro-atherogenic molecule when it is expressed in transgenic mice at a level similar to that in humans: evidence of a potentially relevant species-specific interaction with diet. *J. Lipid Res.* 1998. **39:** 457–462.

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High density lipoprotein cholesterol (HDLc) is inversely related to the risk of atherosclerosis in humans (1). However, not all individuals with HDLc deficiency have the expected cardiovascular risk and the mechanisms by which HDL modulates atherosclerosis susceptibility are poorly defined (1). HDL particles are classified (2) according to the content of their major apolipoproteins (apos), apoA-I and apoA-II. Different classes of HDL may have differential protective effects against atherosclerosis, but clinical studies addressing this issue have yielded conflicting results (1, 2). Genetic modification of experimental animals is a powerful way to dissect the contribution of specific gene products to disease (3). Results from such experiments leave little doubt concerning the anti-atherogenic role of human apoA-I (3–5). However, the role of human apoA-II in atherosclerosis remains poorly defined and controversial. Schultz et al. (4) showed that human apoA-II coexpression with human apoA-I in transgenic mice abrogated the anti-atherogenic effects of apoA-I, whereas

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; HDLc, high density lipoprotein cholesterol; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

[1]To whom correspondence should be addressed.

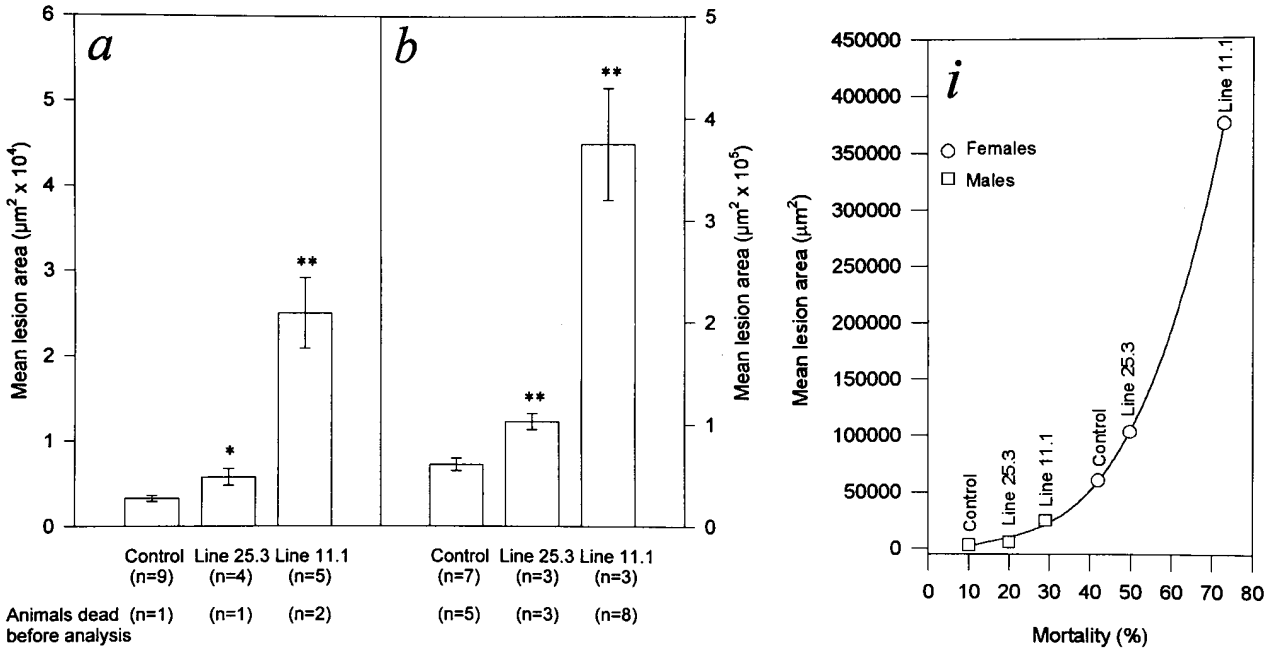

Fig. 1. Atherosclerosis in the proximal aorta of human apoA-II transgenic and control mice after 9 months of an atherogenic diet. *a*: Area of atherosclerosis (expressed per section) in males. *b*: Area of atherosclerosis in females. In *a* and *b*, results are expressed as mean ± SEM; $*P < 0.05$, $**P < 0.001$ compared to control mice (Mann-Whitney U test). The number of mice analyzed and the number of mice dead before the end of the 9-month period is also shown. *c* to *j*: Representative photomicrographs of proximal aortic sections of C57BL/6 mice. All sections were stained with Oil red O and counterstained with hematoxylin; all panels have the same magnification, scale bar = 100 μm. *c*: Flat early fatty streak type I lesions in aortic valve attachments of a male control. *d*: Type I lesion of a 25.3 transgenic male. *e*: Raised lesions in aortic valve attachments extending to the free aortic wall in a 11.1 transgenic male. *f*: Multilayered foam cell deposits in a type II lesion of a control female. *g*: Fibroproliferative type II lesion of a 25.3 transgenic female. *h*: Severe fibroproliferative lesion with a large calcification (arrow) (confirmed by von Kossa method) in the free aortic wall of a 11.1 transgenic female. *i*: Association between the mortality rate before the end of the 9-month period of atherogenic diet and the area of aortic atherosclerosis found in the mice that survived this period and, therefore, could be analyzed.

initial reports from two laboratories suggest that isolated expression of human apoA-II in mice confers a protective effect against atherosclerosis (5, 6). The observation that murine apoA-II transgenic mice are more prone to atherosclerosis than their littermate controls (7, 8) suggests that there are species differences in apoA-II action on atherogenesis. This seems feasible considering the structural and functional differences that exist between human and mouse apoA-II (4, 5, 7–11). An unusual characteristic of the stimulatory effect of murine apoA-II on atherosclerosis is that it is much more pronounced when animals are fed a regular chow diet than when fed a high fat, high cholesterol diet (7, 8). In this report we demonstrate that human apoA-II is, indeed, a pro-atherogenic molecule when it is expressed in mice at a level similar to that in humans (30–35 mg/dl), but only when the animals are fed a high fat, high cholesterol diet. Hence, even though the pro-atherogenic properties of apoA-II are a general characteristic of this molecule, there is a potentially relevant species-specific interaction between human apoA-II expression and diet.

METHODS

Animals

Human apoA-II transgenic mice were developed by microinjection of a 3-kilobase base pair fragment of human genomic DNA into fertilized eggs of C57BL/6 mice (9). Of the three lines obtained, named 25.3, 21.5, and 11.1, we selected the 25.3 and 11.1 for study as their plasma human apoA-II concentrations span that of humans (9). Control and transgenic mice between 2 and 4 months of age were assigned to either an atherogenic diet or to a regular chow diet. After 9 months on the atherogenic diet, the mice that survived were killed. Because of the resistance to atherosclerosis of C57BL/6 mice when maintained on a regular chow diet (Purina mice diet 5001), this diet was extended up to 16 months. The high fat, high cholesterol (atherogenic) diet used (TD 88051 Harlan Teklad, Madison, WI) has the following composition: 75% mouse chow 5015, 7.5% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate.

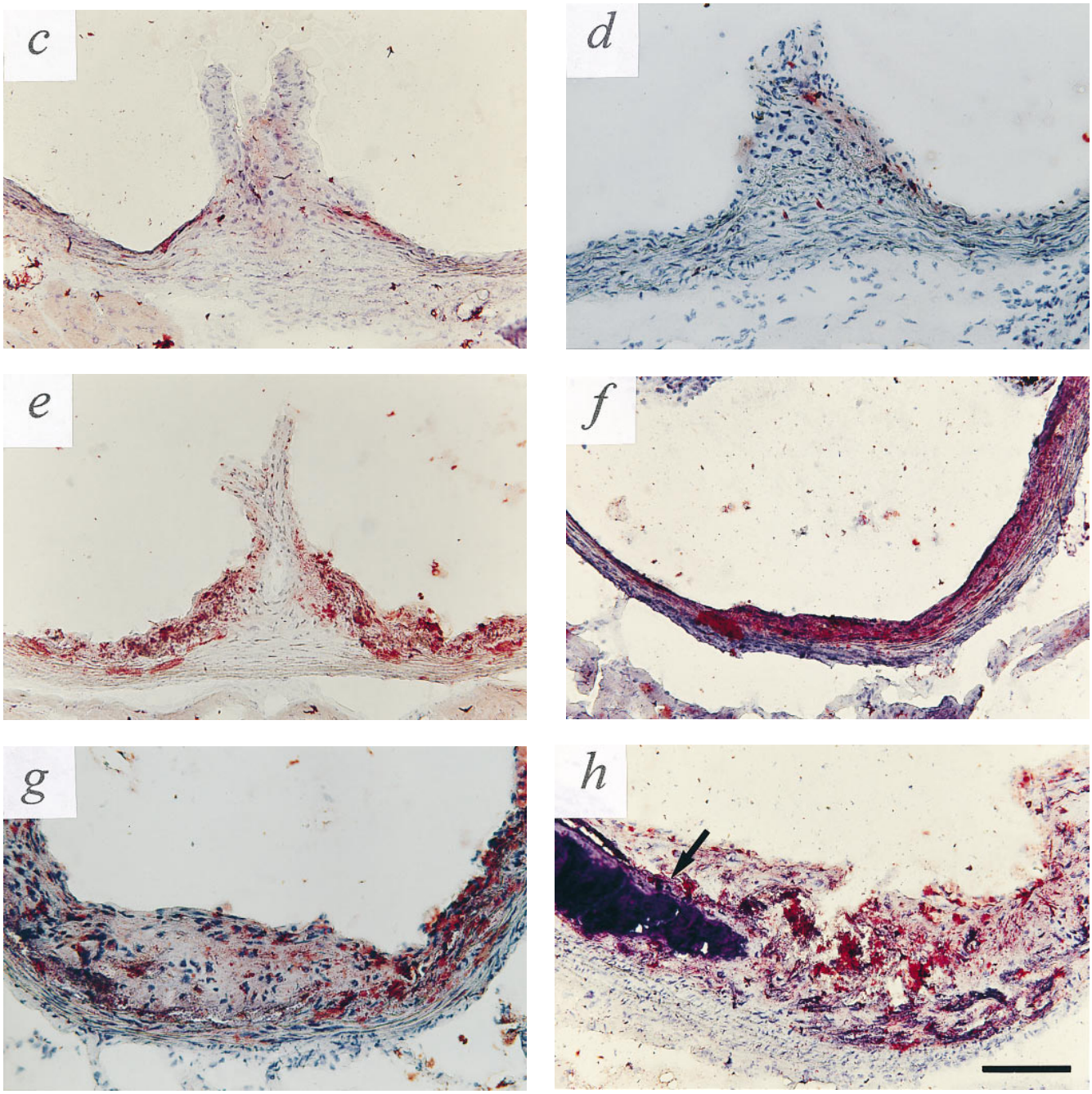

Fig. 1.

Quantification of atherosclerosis

Mice subjected to 9 months of an atherogenic diet were anesthetized and exsanguinated at 11–13 months of age. The hearts were removed, embedded in OCT compound (Tissue-Tek), and sectioned as previously described (12). Oil red O staining of lesion areas present in the proximal aorta were measured in each mouse in eight 10-μm sections interspaced by 40 μm measured with a calibrated eyepiece. The first section was taken 40 μm distal to the point placed between the end of the aortic sinus and the beginning of the aorta. In the case of fibroproliferative lesions, the area quantified included all lesions (from the lumen to the normal vessel wall) and not just the fatty portion. The quantification of atherosclerosis was performed blinded with respect to the origin of the specimens. Also, qualitative analyses were performed in all sections of the proximal aorta as previously published (13).

Determination of lipids, lipoproteins, apolipoproteins, and enzymes

Methodologies for measuring lipid, lipoprotein, and apolipoprotein concentrations as well as endogenous lecithin:cholesterol acyltransferase (LCAT) activity were as described (9, 14). Unlike human, mouse pre-heparin plasma contains high levels of hepatic lipase (HL) with a low affinity for heparin (15). The pre-heparin plasma HDL-triglyceride hydrolysis mediated by HL was measured as free [^{3}H]oleate generated after incubation of a mixture of a pooled isolated [^{3}H] triolein-HDL and the individual pre-heparin plasmas (15). The origin of the pool of radioactive HDL used in the mixtures was the same as that of the pre-heparin plasma (control mice, 25.3 or 11.1) analyzed. The radioactive:non-radioactive HDL ratio in the final mixture was usually about 1:3. Results are expressed as relative to total (radioactive plus non-radioactive) HDL-triglyceride. Electrophoretic mobility of mouse apoA-I in plasma was determined by Western blotting using specific polyclonal antibodies (9).

RESULTS AND DISCUSSION

The results of the studies in transgenic mice expressing plasma levels of human apoA-II similar to those in humans could be relevant to human pathophysiology because apoA-II production rate, rather than its catabolism, is the major factor determining the concentration of apoA-II and the distribution of apoA-I between HDL particles that do, or do not, contain the apoA-II component (16). Moreover, there is evidence that the rate of synthesis of apoA-II in humans may be, largely, under genetic control (17).

After an atherogenic diet, our human apoA-II (25.3 and 11.1) transgenic mice developed more extensive aortic atherosclerosis than control mice (**Fig. 1a** and **1b**). The areas of proximal aortic atherosclerosis in the 25.3 and the 11.1 males were, respectively, 1.8-fold and 7.7-fold that of the control male mice (Fig. 1a). The same comparison in females indicated an increase of the atherosclerosis area of 1.7-fold in 25.3 mice and 6.2-fold in 11.1 mice (Fig. 1b). Hence, there is a clear apoA-II dose-dependent increase in atherosclerosis susceptibility in the transgenic mice. As with other studies in mice, the area of atherosclerosis was greater in females relative to males (7, 13). Representative examples of the lesions observed are presented in Fig. 1 (panels c to h). The high mortality rate of mice in the course of the atherogenic diet is noteworthy (Fig. 1a and 1b). Although the precise cause of death in these animals is not known, the mortality rate of each group seems closely related to the area of aortic atherosclerosis (Fig. 1i). The death of several of these mice was noticed quickly enough to allow the analyses of the aortas. Large and advanced atherosclerotic lesions were observed in these cases. Furthermore, a major accumulation of lipids was sometimes observed in the coronary arteries and, as such, myocardial infarction could well be the cause of mortality in these mice. A high mortality rate has been reported previously in mice fed an atherogenic diet who had occlusions of the coronary arteries (18).

For more detailed comparison, the atherosclerotic lesions were classified in two types (type I are lesions confined to the aortic valves and type II lesions are those that extend to the free aortic wall) and further sub-grouped as flat, raised, and advanced (13). This analysis also demonstrated that transgenic mice developed more advanced atherosclerotic lesions than control mice (data not shown).

In contrast to the atherogenic diet-fed animals, there was no significant difference in the area of atherosclerotic lesion of either transgenic line compared to control mice when they were fed a regular chow diet. These areas, in μm^2/section and expressed as mean $\pm$ SE, were: 283 $\pm$ 111 in control males (n = 5), 625 $\pm$ 196 in 25.3 male transgenic mice (n = 2), and 487 $\pm$ 233 in 11.1 male transgenic mice (n = 4). Area lesions of control females (n = 4), 25.3 transgenic females (n = 4), and 11.1 transgenic females (n = 4) were, respectively, 1110 $\pm$ 460, 209 $\pm$ 57, and 643 $\pm$ 460. The variable influence of human apoA-II transgenesis on atherosclerosis in both types of diet was not due to different human apoA-II plasma levels. None of these 23 mice died before the end of the diet period; suggesting, again, that mice mortality was directly related to atherosclerosis.

The (VLDL-cholesterol + LDL-cholesterol) $\div$ HDLc ratio was lower in male transgenic mice than in controls fed an atherogenic diet; the ratio being higher in female transgenics than in controls of the same sex (**Table 1**). However, the relative increase in the atherosclerotic lesion area of transgenic mice of either gender is similar in relation to their control littermates. It is noteworthy that the HDLc is either higher (in male 25.3 mice), or at least not different, in transgenic compared to control animals, indicating that the absolute HDLc did not play a significant role in determining atherogenicity in these animals. Therefore, at least part of the pro-atherogenic effect of the human apoA-II expression may be due to the different protein composition of HDL in transgenic mice (Table 1). If HDL with apoA-I is more anti-atherogenic than HDL with apoA-I and apoA-II, a higher proportion of the latter particles would be expected to be less protective (3, 4, 7, 8).

There are biologically plausible mechanisms to ex-

plain why the expression of human apoA-II in mice is pro-atherogenic. These mice, especially those of 11.1 line, may have an impaired reverse cholesterol transport. This transgenic line has a reduced LCAT endogenous activity and, conversely, an increased reactivity to HL (Table 1). LCAT and HL have opposite actions in the remodeling of HDL particles: LCAT induces formation of αHDL from preβ HDL, whereas HL action forms preβHDL from αHDL (19). The possibility that 11.1 transgenic mice plasma had a sharp reduction in apoA-I-containing αHDL was confirmed by Western blot analysis (**Fig. 2**). In contrast, mouse and human apoA-II could be detected in α HDL of 11.1 mice concomitantly with a substantial lipid component as judged by Sudan Black B staining (data not shown). It is possible that LCAT was trapped within the αHDL containing the human apoA-II because this apolipoprotein is a poor cofactor for the enzyme (9). The transfer of cholesterol from preβHDL to αHDL was shown by Castro et al. (20) to be slower in human apoA-I/apoA-II than in apoA-I transgenic mice; correlating with a more efficient cholesterol efflux in the latter. However, the report dealt with transgenic mice fed a regular chow diet; a situation in which atherosclerosis does not develop. Formation of preβHDL migrating apoA-I in 11.1 transgenic mice plasma could also be increased as a result of its displacement from HDL by human apoA-II (9).

Previously, murine apoA-II transgenic mice (especially when maintained on regular chow diet) were shown to have increased atherosclerosis susceptibility but in the context of increased concentrations of HDLc and HDL particle size elevation, which is the opposite to their human apoA-II transgenic counterparts (4, 5, 7–11). Taken together, these results strongly support the concept that the pro-atherogenic properties of apoA-II are a general characteristic of this molecule and are not directly related to the HDLc concentration. The mechanisms by which apoA-II expression may cause increased susceptibility to atherosclerosis also include the decrease of the HDL inhibition of LDL oxidative modification (8) and the induction of insulin resistance (21, 22).

In summary, transgenic mice expressing human apoA-II have a dose-related increase of atherosclerotic lesions but only when fed an atherogenic diet. This provides evidence of a species-specific interaction between the expression of human ApoA-II and an atherogenic diet which could be of considerable potential relevance as the high fat diets are a common occurrence in developed countries. If the findings of this study are confirmed, clarification of the mechanisms implicated will require detailed characterization of the effects of human apoA-II expression on reverse cholesterol transport, on protection of LDL modification, and on insulin sensitivity.

TABLE 1. Plasma concentrations of lipids, lipopoproteins, apolipoproteins and activities of HDL-modifying enzymes in human apoA-II transgenic mice and control littermates after 9 months of an atherogenic diet

Analyte	Control C57BL/6	Line 25.3	Line 11.1
Males	n = 9	n = 4	n = 5
Total cholesterol	227 ± 11	234 ± 14	153 ± 11[c]
VLDL-cholesterol	120 ± 8	77 ± 11[a]	70 ± 2[c]
LDL-cholesterol	40 ± 8	50 ± 6	29 ± 5
HDL-cholesterol	67 ± 4	104 ± 3[b]	54 ± 7
Free cholesterol (%)	18 ± 1	24 ± 2[a]	21 ± 2[a]
Triglycerides	18 ± 3	ND	48 ± 14
Mouse apoA-I	117 ± 3	121 ± 6	50 ± 5[c]
Mouse apoA-II	36 ± 2	38 ± 1	22 ± 2[c]
Human apoA-II	0 ± 0	24 ± 4[c]	91 ± 6[c]
Mouse apoE	7 ± 0.5	7 ± 0	8 ± 4
Hepatic lipase (μm/h)	67 ± 4	93 ± 13	120 ± 4[c]
LCAT (μm/h)	153 ± 25	ND	48 ± 13[c]
Females	n = 7	n = 3	n = 3
Total cholesterol	280 ± 17	295 ± 43	350 ± 45
VLDL-cholesterol	115 ± 10	130 ± 30	187 ± 31
LDL-cholesterol	75 ± 10	83 ± 7	100 ± 28
HDL-cholesterol	90 ± 8	76 ± 11	62 ± 18
Free cholesterol (%)	25 ± 2	20 ± 1	35 ± 4[a]
Triglycerides	7 ± 2	8 ± 3	64 ± 25[b]
Mouse apoA-I	117 ± 17	70 ± 3	40 ± 6[b]
Mouse apoA-II	30 ± 6	18 ± 1	17 ± 1
Human apoA-II	0 ± 0	23 ± 3[b]	101 ± 12[b]
Mouse apoE	23 ± 11	7 ± 0.3	24 ± 16
Hepatic lipase (μm/h)	72 ± 6	83 ± 6	119 ± 4[a]
LCAT (μm/h)	184 ± 7	ND	37 ± 9[a]

Unless otherwise specified, results are expressed as mg/dl. Data are shown as mean ± SEM.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$ versus control mice (Mann-Whitney U test); ND, not determined.

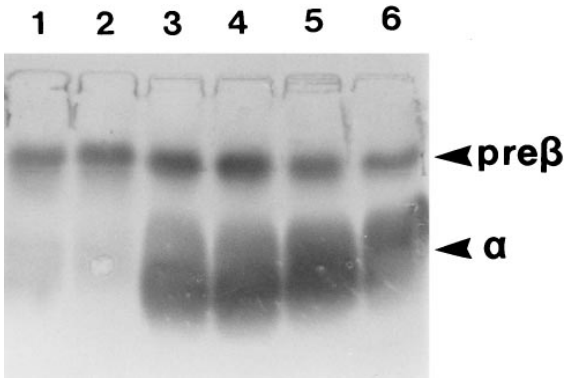

Fig. 2. Western blot of plasmas obtained from mice after 9 months of an atherogenic diet, probed with polyclonal antibodies to mouse apoA-I. Lanes 1 and 2: 11.1 transgenic male and female mice; lanes 3 and 4: control male and female mice; lanes 5 and 6: 25.3 transgenic male and female mice.

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