In vivo interactions of apoA-II, apoA-I, and hepatic lipase contributing to HDL structure and antiatherogenic functions

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Abstract **Studies with mice have revealed that increased expression of apolipoprotein A-II (apoA-II) results in elevations in high density lipoprotein (HDL), the formation of larger HDL, and the development of early atherosclerosis. We now show that the increased size of HDL results in part from an inhibition of the ability of hepatic lipase (HL) to hydrolyze phospholipids and triglycerides in the HDL and that the ratio of apoA-I to apoA-II determines HDL functional and antiatherogenic properties. HDL from apoA-II transgenic mice was relatively resistant to the action of HL in vitro. To test whether HL and apoA-II influence HDL size independently, combined apoA-II transgenic/HL knockout (HLko) mice were examined. These mice had HDL similar in size to apoA-II transgenic mice and HLko mice, suggesting that they do not increase HDL side by independent mechanisms. Overexpression of apoA-I from a transgene reversed many of the effects of apoA-II overexpression, including the ability of HDL to serve as a substrate for HL. Combined apoA-I/apoA-II transgenic mice exhibited significantly less atherosclerotic lesion formation than did apoA-II transgenic mice. These results were paralleled by the effects of the transgenes on the ability of HDL to protect against the proinflammatory effects of oxidized low density lipoprotein (LDL). Whereas nontransgenic HDL protected against oxidized LDL induction of adhesion molecules in endothelial cells, HDL from apoA-II transgenic mice was proinflammatory. HDL from combined apoA-I/apoA-II transgenic mice was equally as protective as HDL from nontransgenic mice. Our data suggest that as the ratio of apoA-II to apoA-I is increased, the HDL become larger because of inhibition of HL, and lose their antiatherogenic properties.** —Hedrick, C. C., L. W. Castellani, H. Wong, and A. J. Lusis. **In vivo interactions of apoA-II, apoA-I, and hepatic lipase contributing to HDL structure and antiatherogenic functions.** ***J. Lipid Res.*** **2001.** 42: **563–570.**

Supplementary key words atherosclerosis • lipoprotein • paraoxonase • transgenic mice

Epidemiological studies have shown that plasma levels of high density lipoproteins (HDL) are inversely associated with risk of coronary artery disease (CAD) (1–3). HDL may protect against CAD by removing cholesterol from peripheral tissues and transporting it to the liver, a process termed "reverse cholesterol transport" (1–3). In addition, HDL may protect against CAD by inhibiting low density lipoprotein (LDL) oxidation within the artery wall (4). The two major apolipoproteins associated with HDL are apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II). In humans, apoA-I and apoA-II constitute approximately 60% and 20%, respectively, of the protein mass of HDL (1, 2). ApoA-I is a primary structural protein for HDL and also serves as a cofactor for the enzyme lecithin:cholesterol acyltransferase (1), and elevated levels of apoA-I are associated with decreased risk of CAD (5). In several animal models of atherosclerosis, apoA-I overexpression increased HDL levels and reduced the development of atherosclerosis (5–7).

The role of apoA-II in HDL function, on the other hand, is unclear. Unlike apoA-I, apoA-II is not required for assembly of HDL because members of a family with apoA-II deficiency exhibited approximately normal levels of plasma lipoproteins (8). However, there is evidence that apoA-II influences several aspects of HDL metabolism, including reverse cholesterol transport (9), HDL composition (10), HDL levels (10–13), and the ability of HDL lipids to serve as substrates for hepatic lipase (HL) (10). We have previously shown that overexpression of mouse apoA-II in transgenic mice promoted the development of atherosclerosis on both chow and atherogenic diets despite causing significant elevations in plasma HDL cholesterol (HDL-Chol) levels (11). The HDL in the apoA-II transgenic mice also

Abbreviations: apoA-I, apolipoprotein A-I; CAD, coronary artery disease; FPLC, fast protein liquid chromatography; HAEC, human aortic endothelial cells; HDL-Chol, high density lipoprotein cholesterol; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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tended to be significantly larger (12) and were proinflammatory in a coculture model of the artery wall (14).

In the present study we have examined mechanisms resulting in the altered structure and function of HDL in mice overexpressing apoA-II. We hypothesized that the increased size of HDL in the transgenic mice resulted from inhibition of HL activity, because HL knockout (HLko) mice exhibited increased HDL-Chol levels as well as increased HDL size (15). This hypothesis was tested by examining combined apoA-II transgenic/HLko mice, and the results are consistent with our hypothesis. We also hypothesized that the ratio of apoA-I to apoA-II is important for HDL structure and HDL function relevant to the development of atherosclerosis. To test this, we examined HDL in transgenic mice overexpressing apoA-I, apoA-II, or both. We observed that the ratio of apoA-I to apoA-II influences atherosclerotic lesion development, the anti-inflammatory properties of HDL, the size of HDL, and the ability of HDL lipids to be hydrolyzed by HL.

EXPERIMENTAL PROCEDURES

Mice

ApoA-II transgenic mice (mAIItg) were constructed with a mouse apoA-II genomic clone as described previously (12). ApoA-I transgenic mice overexpressing the human apoA-I gene (hAItg) were kindly provided by J. Breslow (Rockefeller University, New York, NY). Inbred strain C57BL/6J (B6) mice and HLko mice generated by gene targeting (15) were purchased from the Jackson Laboratories (Bar Harbor, ME). Both the AItg mice and the AIItg mice were backcrossed to C57BL/6J mice for 10 or more generations to avoid problems resulting from a mixed genetic background. The combined apoA-I/apoA-II (hAItg/ mAIItg) transgenic mice were created by crossing AItg mice with AIItg mice and selecting progeny homozygous for both transgenes. The presence of the apoA-II transgene was identified by analysis of the apoA-II isoelectric focusing pattern because the apoA-II expressed from the Swiss mouse apoA-II genomic clone carries a more basic apoA-II allele than the C57BL/6J apoA-II allele (11). HAItg mice were identified by determining human apoA-I concentration in plasma by rate nephelometry, using a polyclonal antibody to human apoA-I (Beckman, Fullerton, CA). HLko mice were identified by polymerase chain reaction as previously described (15). Similarly, combined apoA-IItg-HLko (mAIItg/HLko) mice were generated by crossing HLko mice with mAIItg mice, followed by backcrossing these to HLko mice and selecting progeny homozygous for the HL-targeted allele and containing the apoA-II transgene. Male mice were used in all studies.

Mouse aortic lesion analysis

Transgenic and control mice were maintained on a Purina chow diet containing 4.5% fat until 4–6 months of age, when blood was collected after an overnight fast. Transgenic and control mice were then fed an atherogenic diet (Harlan Teklad, Indianapolis, IN) containing 1.25% cholesterol, 7.5% cocoa butter, and 0.5% cholic acid (with a total fat content of 15%) for 15 weeks, when they were bled, and killed by cervical dislocation under isofluorane anesthesia. The size of aortic fatty streak lesions in 10-mm-thick sections of the proximal aorta, from the aortic valves to the arch, was determined as previously described (16).

Plasma lipoprotein measurements

Plasma samples from mice fasted overnight were obtained by retro-orbital bleeding under isofluorane anesthesia. Plasma lipids were determined by enzymatic procedures with minor modifications as described previously (11, 12). HDL-Chol was determined after precipitation of very low density lipoprotein (VLDL) and LDL with heparin and manganese chloride. Combined VLDL cholesterol plus LDL cholesterol concentrations were calculated by subtracting the HDL-Chol value from the total cholesterol value. Each sample was measured in triplicate with an external control sample with known analyte concentration included for each assay.

Plasma apolipoprotein determinations

Plasma apolipoprotein concentrations were determined by a combination of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting according to modifications of previously described procedures (11, 12). Plasma samples were diluted 1:25 in sample buffer [10% 2-mercaptoethanol, 0.25 M Tris-HCl (pH 6.8), 0.2% sodium dodecyl sulfate, 20% glycerol, 0.025% bromthymol blue] and 20 ml of each sample preparation was loaded onto 4–20% polyacrylamide Tris-glycine gels (Novex, San Diego, CA). SDS-PAGE was performed at 45 mA for 1.5 h, and proteins were transferred to nitrocellulose with a semidry blotter (Bio-Rad, Hercules, CA), at 14 V for 30 min. Protein bands were visualized by a chemiluminescence procedure and quantitated by densitometry (SciScan 5000; United States Biochemical, Cleveland, OH). The resulting signals were shown to be in the linear portion of the response curve, using apolipoprotein standards run in parallel at various dilutions (12). Human apoA-I in plasma was quantitated by rate nephelometry on a Beckman array 360.

Antibodies

Monospecific polyclonal antibodies prepared in rabbits to mouse apoA-I and mouse apoA-II were utilized (12, 13, 17). Quantitative estimates of the levels of the apolipoproteins were performed as previously described using apolipoproteins isolated from mouse lipoproteins (12, 13, 17). Antibodies to human apoA-I were purchased from Beckman (11, 12).

Gel-filtration chromatography

Plasma lipoproteins were fractionated by fast protein liquid chromatography (FPLC), using two Superose 6 columns connected in series (Amersham Pharmacia Biotechnology, Piscataway, NJ). Fractions of 0.5 ml were collected at a rate of 0.5 ml/min. Plasma lipoprotein separations achieved by this procedure were highly reproducible, and chromatograms from duplicate runs appeared nearly identical. Cholesterol was measured in each fraction by enzymatic assay as described above. HDL particle sizes were determined by comparing elution volumes with molecular weight standards.

Nondenaturing gradient PAGE

Nondenaturing PAGE was performed as described (13). Gels containing a 4–12% linear polyacrylamide gradient (Novex) were electrophoresed at 200 V for 90 min in 90 mM Tris, 80 mM boric acid, and 3 mM ethylenediaminetetraacetic acid, pH 8.4. Before electrophoresis, 10 μl of plasma samples was stained for lipid by incubation at 4°C overnight with 8 μl of freshly prepared Sudan Black B dye solution (5 parts 1% Sudan Black B in 100% ethylene glycol:3 parts 40% sucrose).

HL activity determinations

Total HL activity in plasma of mice was quantitated by a stable, radioactive substrate emulsion assay as described by Nilsson-Ehle and Schotz (18). Nonheparinized plasma was used in this study because in mice approximately two-thirds of HL is present in the

TABLE 1. Plasma lipid levels in male transgenic mice fed a chow diet

Group	Total Chol	HDL-Chol	Triglyceride	ApoA-I: ApoA-II Ratio	Human ApoA-I	Mouse ApoA-I	Mouse ApoA-II	Plasma HL Activity
	mg/dl	*mg/dl*	*mg/dl*	(*w/w*)	*mg/dl*	*mg/dl*	*mg/dl*	*nmol/min/ml*
B6 control (n = 15)	87 ± 11^a	55 ± 2^a	44 ± 6^a	5.8	0	128 ± 5^a	22 ± 3^a	63 ± 4^a
hAItg (n = 13)	136 ± 7^b	126 ± 7^b	38 ± 8^a	13.3	303 ± 18^a	72 ± 10^b	28 ± 5^a	56 ± 4^a
mAIItg (n = 10)	136 ± 18^b	125 ± 20^b	118 ± 18^b	1.6	0	105 ± 11^a	64 ± 7^b	67 ± 6^a
hAItg/mAIItg (n = 9)	189 ± 30^c	180 ± 29^c	138 ± 36^b	7.5	383 ± 32^a	59 ± 6^b	59 ± 7^b	65 ± 5^a

Values represent means ± SEM. Within each column, values with different letters are significantly different for each group by ANOVA, $P < 0.001$. HDL, high density lipoprotein; ApoA-I, apolipoprotein A-I; HL, hepatic lipase.

plasma (19). HL activity was determined in plasma that had been stored at −70°C, using radioactive emulsified [^{3}H]triolein in 1 M NaCl as the substrate; no other plasma lipase is active under these conditions. Activity was determined at two concentrations to ascertain linearity in the assay.

Lipid hydrolysis determinations

HDL from each group of mice was isolated from pooled plasma samples by density ultracentrifugation (d = 1.063–1.21 g/ml) as described (20). The hydrolysis of HDL lipids from transgenic mice was examined as described by Mowri et al. (21). Briefly, HDL from AItg mice, AIItg mice, AItg/AIItg mice, and C57BL/6J control mice (40 μg or protein for each sample) were incubated with 1.5 μg of recombinant human HL for 1, 2, and 4 h at 28°C in 50 μl of 100 mM Tris-HCl, pH 8.0, containing 4% (w/v) bovine serum albumin. An additional 40 mg of each HDL preparation was also added to 1.5 mg of recombinant HL and immediately stored at 4°C to use as time 0 controls. The hydrolysis and release of free fatty acids (FFA) from triglycerides and phospholipids in HDL were quantitated by a colorimetric assay as described (11, 12).

Monocyte adhesion assay for oxidized LDL

Human aortic endothelial cells (HAEC) were grown to confluence in M199 medium supplemented with 10% fetal bovine serum (FBS) with endothelial cell growth supplement and heparin. Minimally oxidized LDL (MM-LDL, 250 μg/ml, prepared as described) (22) and 150 μg of HDL from each transgenic mouse group were mixed and added in M199-5% FBS to the HAEC monolayer, and incubated at 37°C for 4 h. Adhesion assays were then performed as described (23). Briefly, cells were rinsed twice with serum-free medium, and 1×10^6 monocytes were incubated with the HAEC for 15 min at 37°C. Nonadherent monocytes were rinsed away with phosphate-buffered saline and cells were fixed with 1% glutaraldehyde. The number of attached monocytes was counted with a phase-contrast microscope under ×10 magnification. Blood monocytes were obtained from a large pool of healthy donors by modification of the Recalde method (24).

Statistical analysis

All data are represented as means ± SEM. Statistical analysis was performed by using the Student's *t*-test. When multiple comparisons were evaluated, analysis of variance (ANOVA) was performed. For the ANOVA, a Fisher exact test was employed to determine 95% confidence intervals.

RESULTS

Size and levels of HDL from mice overexpressing apoA-I, apoA-II, or both

In agreement with previous studies, overexpression of mouse apoA-II or human apoA-I in transgenic mice resulted in elevated HDL-Chol levels (**Table 1**). Combined hAItg/mAIItg transgenic mice had still higher levels of HDL-Chol (Table 1, **Fig. 1**). The effect of overexpression of

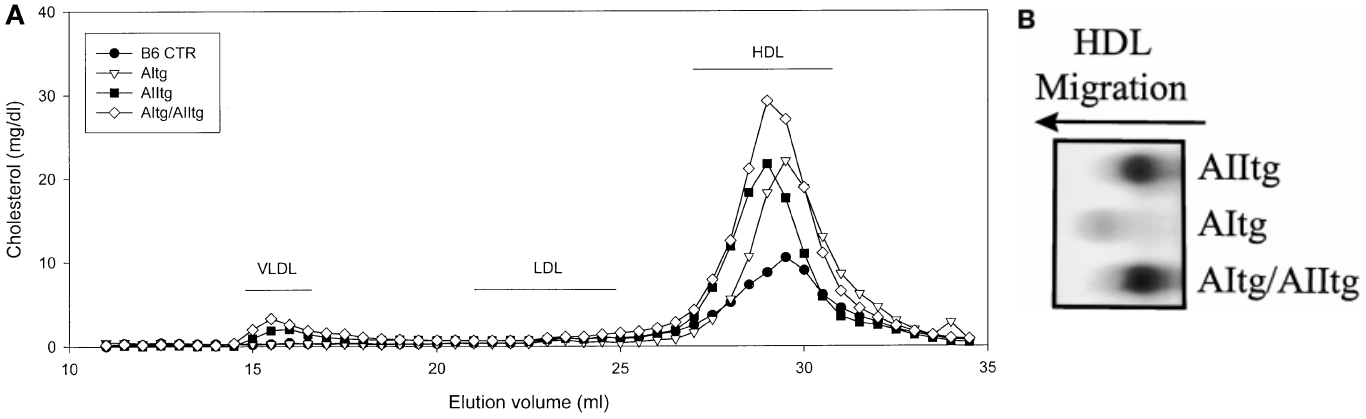

Fig. 1. Mice overexpressing apoA-II have larger high density lipoproteins (HDL) than do control mice or mice overexpressing apolipoprotein A-I (apoA-I). A: Lipoproteins from pooled plasma of groups of transgenic mice were separated by fast protein liquid chromatography (FPLC) as described in Materials and Methods. The approximate elution volumes of very low density lipoproteins (VLDL), low density lipoproteins (LDL), and HDL are indicated. Plasma samples were from C57BL/6J control mice (solid circles), mAIItg mice (solid squares), hAItg mice (open inverted triangles), or combined hAItg/mAIItg mice (open diamonds). B: Pooled plasma samples from three mice per group were analyzed by nondenaturing gradient gel electrophoresis and HDL lipid was stained with Sudan Black B as described in Materials and Methods. Shown are the HDL from each group stained for lipid.

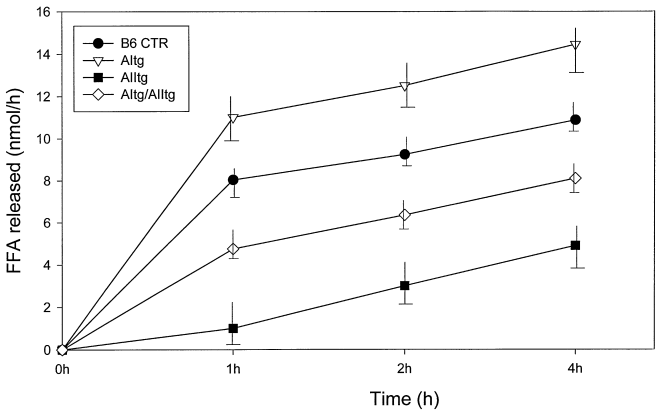

Fig. 2. HDL enriched with apoA-II is a poor substrate for hepatic lipase (HL). HDL from each group of mice was incubated with recombinant human HL in vitro as described in Materials and Methods. The hydrolysis of phospholipids and triglycerides from HDL was measured as the release of free fatty acids (FFA). HDL was isolated from hAItg mice (open triangles), mAIItg mice (solid squares), combined hAItg/mAIItg mice (open diamonds), and C57BL/6J control mice (solid circles).

apoA-II and apoA-I on the size of HDL particles was distinct, however. As observed after fast protein liquid chromatography (FPLC) fractionation (Fig. 1A) and gel electrophoresis (Fig. 1B), apoA-II overexpression clearly increased the average size of HDL particles [about 11.5 nm in mAIItg mice compared with about 9.1 nm in B6 control mice (see Fig. 3B for B6 control mice)] whereas apoA-I overexpression slightly decreased the average size of HDL particles (to about 8.9 nm). The combined hAItg/mAIItg mice exhibited an average HDL size slightly smaller than that of the mAIItg mice.

HDL enriched in apoA-II is a poor substrate for HL

One mechanism by which enrichment of HDL for apoA-II could result in larger HDL particles is by decreased HL hydrolysis of HDL triglyceride and phospholipid. To test this, we incubated HDL from the different transgenic mice with recombinant human HL and measured the release of FFA from the HDL over a 4-h time interval. As shown in **Fig. 2**, HDL that was enriched in apoA-II had decreased release of FFA. The ratio of apoA-I to apoA-II was correlated with the ability of the HDL to serve as a substrate for HL. HDL from mAIItg mice was significantly less effective as a substrate for HL than were HDL from hAItg/mAIItg mice. All groups of the transgenic mice had similar levels of plasma HL activity, which averaged approximately 63 ± 4 nmol of FFA per min per ml for each group. These data suggested that the apoA-I-to-apoA-II ratio on HDL influences the interaction of HDL with HL and indicated that apoA-II-enriched HDL served as poor substrates for HL.

Increase in HDL size in apoA-II transgenic mice results from inhibition of HL

To study the influences of apoA-II on HDL size, we combined HL null and apoA-II transgenic mice (mAIItg/HLko). As previously observed, HLko mice have large HDL comparable to human HDL_1 (15) and similar in size to mAIItg HDL (**Fig. 3**). If the mechanism by which apoA-II increases the size of HDL involves the inhibition of HL, the HDL of combined mAIItg/HLko mice should be similar in size to that of HLko mice and mAIItg mice. If HL and apoA-II influence HDL size by different mechanisms, the HDL particles in combined mAIItg/HLko mice should be larger than those of either mAIItg or HLko mice. Indeed, as judged by the peak elution volume of HDL-Chol in FPLC (Fig. 3A), the HDL from the combined mAIItg/HLko mice was similar in size to HDL from either the mAIItg mice or HLko mice, and all three groups had HDL that was larger than B6 control HDL. Similar data were obtained for the mAIItg/HLko HDL after native gel electrophoresis and lipid staining with Sudan Black B (Fig. 3B). These data indicate that apoA-II and HL act, in part, through the same

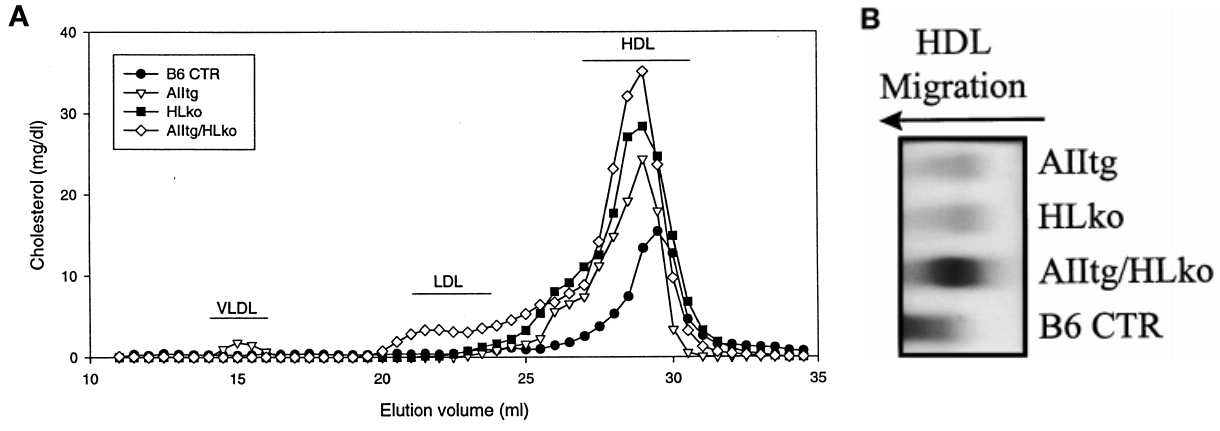

Fig. 3. Increase in HDL size in apoA-II transgenic mice results from an inhibition of HL. A: Lipoproteins from pooled plasma of groups of transgenic mice were separated by FPLC as described in Materials and Methods. Plasma samples were from mAIItg mice (open inverted triangles), HLko mice (solid squares), combined mAIItg/HLko mice (open diamonds), and C57BL/6J control mice (solid circles). B: Pooled plasma from four mice per group was analyzed by nondenaturing gradient gel electrophoresis and HDL lipid was stained with Sudan Black B as described in Materials and Methods. Shown is the HDL from each group stained for lipid.

TABLE 2. Plasma lipid levels in male combined mAIItg/HL knockout (HLko)mice fed a chow diet

Group	Total Chol	Triglycerides	HDL-Chol	FFA	Plasma HL Activity
	mg/dl	*mg/dl*	*mg/dl*	*mg/dl*	*nmol/min/ml*
B6 control (n = 5)	71 ± 4^{a}	41 ± 9^{a}	65 ± 5^{a}	67 ± 10^{a}	63 ± 4^{a}
HLko (n = 6)	187 ± 37^{b,c}	100 ± 24^{b}	142 ± 31^{b,c}	131 ± 10^{b}	ND
mAIItg (n = 11)	140 ± 20^{b}	110 ± 19^{b}	125 ± 15^{b}	152 ± 10^{b}	66 ± 5^{b}
mAIItg/HLko (n = 6)	217 ± 36^{c}	97 ± 23^{b}	171 ± 24^{c}	147 ± 18^{b}	67 ± 4^{b}

Values represent means ± SEM. Within each column, values with different letters are significantly different for each group by ANOVA, $P < 0.003$. ND, None detected.

pathway to influence HDL size in mice, and that the inhibition of HL by apoA-II is responsible, at least in part, for the larger HDL observed in apoA-II-overexpressing mice. The combined mAIItg/HLko mice appeared to have slightly elevated HDL-Chol as compared with mAIItg or HLko mice (**Table 2**, Fig. 3), although this was not significant ($P > 0.05$). It is interesting that the levels of FFA were elevated in plasma of both HLko and mAIItg mice (Table 2), although the mechanism involved is unknown.

Antiatherogenic properties of HDL depend in part on the ratio of apoA-I to apoA-II

We and others have previously observed that apoA-II transgenic mice develop larger fatty streak lesions when fed a high fat diet than do nontransgenic littermates (11). To test whether these effects are due to the elevated levels of apoA-II or the ratio of apoA-I to apoA-II, we compared fatty streak lesion formation in hAItg, mAIItg, combined hAItg/mAItg, and control C57BL/6J mice fed a high fat diet. The levels of plasma apolipoproteins and lipids in these mice are summarized in **Table 3**. The effects of the transgenes on HDL levels are similar to those observed in mice fed a chow diet. As previously observed (5), overexpression of apoA-I protected against lesion development, whereas overexpression of apoA-II increased lesion development, (**Fig. 4**). The combined hAItg/mAIItg mice exhibited lesion development that was significantly less than that of mAIItg mice ($P < 0.05$), although the apoA-II levels of the combined hAItg/mAIItg mice were similar to those of the mAIItg mice (Table 1). It is possible that hypertriglyceridemia contributes in part to increased lesion formation in these mice. However, it is noteworthy that the levels of triglyceride-rich lipoproteins in the combined hAItg/mAIItg mice were at least as high as that of mAIItg mice, yet the hAItg/mAIItg mice exhibited no increase in lesion development compared with control C57BL/6J mice.

Antioxidant properties of HDL depend in part on the ratio of apoA-I to apoA-II

One mechanism by which HDL protects against atherosclerosis appears to be related to its antioxidant functions (3, 4). We previously reported that, whereas HDL from C57BL/6J mice is able to inhibit the inflammatory properties of MM-LDL, the HDL from AIItg mice is actually proinflammatory (14). We have now examined antioxidant properties of HDL from combined hAItg/mAIItg mice. The activity of the oxidized LDL was assessed by examining its ability to induce monocyte binding to cultures of endothelial cells (**Fig. 5**). Consistent with our previous results with a coculture system, we observed that the HDL from control C57BL/6J mice and from hAItg mice reduced monocyte binding to HAEC by MM-LDL ($P = 0.0001$), whereas HDL from mAIItg mice promoted monocyte binding ($P = 0.01$; Fig. 5). HDL from combined hAItg/mAIItg mice was able to inhibit monocyte binding to about the same extent as HDL from C57BL/6J mice or hAItg mice ($P = 0.0001$; Fig. 5). Thus, the anti-inflammatory properties of HDL appear to be a function of the ratio of apoA-I to apoA-II rather than of the absolute levels of each.

DISCUSSION

Studies with transgenic mice carrying the transgene for either human (25) or mouse apoA-II (11) have shown that apoA-II promotes atherogenesis. One of the mechanisms involved is likely to relate to the antioxidant properties of the HDL; thus, whereas the HDL from nontransgenic mice is

TABLE 3. Plasma apolipoprotein and lipid levels in male transgenic mice fed an atherogenic diet for 15 weeks

Group	Total Chol	HDL-Chol	Triglyceride	ApoA-I: ApoA-II Ratio	Human ApoA-I	Mouse ApoA-I	Mouse ApoA-II
	mg/dl	*mg/dl*	*mg/dl*	*(w/w)*	*mg/dl*	*mg/dl*	*mg/dl*
B6 control (n = 16)	274 ± 41^{a}	53 ± 10^{a}	53 ± 6^{a}	5.0	0	131 ± 5^{a}	26 ± 5^{a}
hAItg (n = 19)	260 ± 12^{a}	94 ± 4^{b}	61 ± 8^{a}	14.2	297 ± 28^{a}	74 ± 13^{b}	26 ± 8^{a}
mAIItg (n = 27)	304 ± 23^{b}	109 ± 8^{b,c}	94 ± 18^{b}	1.8	0	136 ± 12^{a}	75 ± 2^{b}
hAItg/mAIItg (n = 10)	347 ± 12^{b}	131 ± 11^{c}	170 ± 36^{c}	6.5	389 ± 24^{b}	62 ± 8^{b}	69 ± 14^{b}

Values represent means ± SEM. Within each column, values with different letters are significantly different for each group by ANOVA, $P < 0.001$.

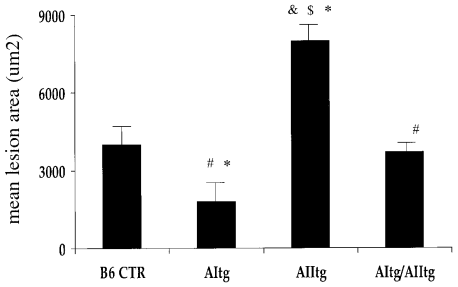

Fig. 4. The ratio of apoA-I to apoA-II in HDL is a determinant of atherosclerosis. Control C57BL/6J mice (B6 CTR, n = 10), hAItg mice (n = 8), mAIItg mice (n = 12), or hAItg/mAIItg mice (n = 10) were maintained on a high fat/high cholesterol diet for 15 weeks, at which point they were killed and the area of oil red O-staining lesions in the proximal aorta was determined. * $P < 0.05$, significantly different from B6 control mice; $ $P < 0.05$, significantly different from hAItg; # $P < 0.01$, significantly different from mAIItg; & $P < 0.01$, significantly different from hAItg/mAIItg.

able to inhibit LDL oxidation, the HDL from apoA-II transgenic mice is actually proinflammatory (14). One of the enzymes involved in the antioxidant properties of HDL is serum paraoxonase and we previously showed that the concentration of paraoxonase is reduced about 40% in HDL of mAIItg mice (14). This reduction, however, is unlikely to fully explain the proinflammatory properties of mAIItg HDL, because HDL from heterozygous serum paraoxonase knockout mice, in which serum paraoxonase activity is reduced by 50%, are still protective against LDL oxidation (26). In addition, serum paraoxonase levels were not significantly increased in apoA-I transgenic mice (27, 28) (Fig. 5). There are several metabolic pathways that can contribute to the atherosclerosis development observed in mAIItg mice. Specifically, the increased levels of VLDL and hypertriglyceridemia observed in these animals (12) could be proatherogenic. Some of the mechanisms that cause hypertriglyceridemia that could contribute to atherogenesis [such as alterations in apoC-III or lipoprotein lipase (LPL) expression or function, or diabetes, obesity, and insulin resistance syndromes] may be present in these animals. We have not yet

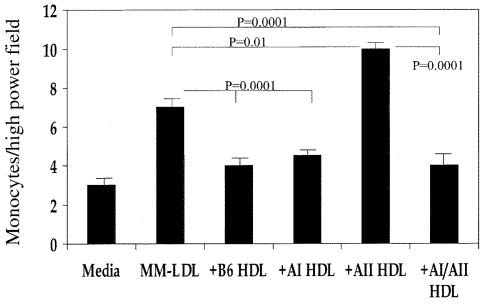

Fig. 5. The ratio of apoA-I to apoA-II is a determinant of the anti-inflammatory properties of HDL. The binding of monocytes to human aortic endothelial cells was quantitated after treatment with MM-LDL in the presence of HDL isolated from control C57BL/6J mice (+B6 HDL), mAIItg HDL (+AII HDL), hAItg HDL (+AI HDL), or combined hAItg/mAIItg HDL (+AI/AII HDL). Plasma HDL from five mice per group were pooled and analyzed. These data represent the mean ± SEM of three experiments.

examined these parameters in these mice. In the current study, we chose to examine HDL metabolism and its role in atherogenesis. In an effort to better understand the functions of apoA-II, we have examined mechanisms contributing to functional and structural alterations in HDL from mAIItg mice. Two conclusions have emerged from these studies. First, the effect of apoA-II on HDL size appears to be due at least in part to reduced hydrolysis of lipids in apoA-II-rich HDL by HL. Second, the effects of apoA-II overexpression on atherogenesis and the antioxidant properties of HDL can be reversed by overexpression of apoA-I, indicating that one important parameter of HDL function is the ratio of apoA-I to apoA-II. These points are discussed below.

The effects of apoA-II on HDL size in mice were first observed in studies of naturally occurring strains. Among inbred strains of mice, the levels of apoA-II range from about 3 mg/dl (in strain SM/J) to about 30 mg/dl (in strain BALB/cJ) (29, 30). Among the strains, the sizes of HDL increased continuously as the levels of apoA-II increased, and the causal relationship between apoA-II expression and HDL size was established in genetic studies showing that apoA-II expression segregates with HDL size (13, 29, 30). Subsequently, the effect of apoA-II overexpression on HDL size was confirmed in transgenic mice that express still higher levels of apoA-II (10–12). Consistent with these studies are data by Breslow and colleagues (31), in which HDL levels and HDL size were reduced in mice in which the apoA-II gene was targeted.

ApoA-II appears to affect HDL size by reducing its ability to act as a substrate for HL. There is no difference in plasma HL activity in any of the mouse groups studied. This is not surprising, however, in that there are no reports of apoA-II levels modulating plasma HL activity in vivo. Breslow and colleagues found no differences in plasma HL activity in either A-II knockout mice (31, 32) or in A-II transgenic mice (10). Although the activity of HL in transgenic mouse plasma was unchanged, the ability of HL to act on apoA-II-enriched HDL in vitro was reduced (Fig. 2). HDL with higher ratios of apoA-II to apoA-I showed less hydrolysis of triglyceride and phospholipid by HL (Fig. 2). To examine whether the increased size of HDL in mAIItg mice was mediated by HL, we generated combined mAIItg/HLko mice. These combined mAIItg/HLko mice exhibited large HDL and elevated HDL-Chol levels, similar to what is found in mAIItg mice. These data suggest that apoA-II reduces the ability of HDL lipids to act as a substrate for HL both in vivo as well as in vitro. Although other parameters of HDL can influence HL activity, our results are consistent with in vitro studies showing that human apoA-II reduces the hydrolysis of HDL phospholipids and triglycerides (10). Also consistent with our results was the finding that HDL from apoA-I knockout mice, consisting largely of apoA-II as the protein component, was relatively resistant to lipolysis by HL (31). Several studies have shown that apoA-II-enriched VLDL inhibits LPL activity (33, 34). Magill et al. (33) found that the VLDL fractional catabolic rate was directly proportional to LPL activity and inversely related to apoA-II levels. Kalopissis and colleagues (35) have shown that apoA-II-enriched VLDL in

transgenic mice resulted in decreased VLDL catabolism by LPL. A variety of genetic epidemiologic studies have indicated an association between an HL gene polymorphism and HDL levels (36, 37), and polymorphisms of the apoA-II gene locus have been associated with both apoA-II levels and the ratio of apoA-II to apoA-I (38–40). Finally, a metabolic study with human volunteers concluded that the levels of apoA-I, apoA-II, and HL could explain a large fraction of the variation in HDL-Chol levels (37). Taken together, these data suggest that there are interactions among apoA-I, apoA-II, and HL that influence the size and levels of HDL-Chol.

ApoA-II also appears to influence some functions of HDL that are relevant to atherosclerosis. Studies with naturally occurring variations of apoA-II in mice first suggested that elevated HDL levels increase HDL-Chol but promote atherosclerosis (29). This was subsequently confirmed with transgenic mice overexpressing mouse (11) or human (25) apoA-II and in mice in which both the HL and apoA-II genes were targeted (31). HDL enriched in apoA-II lacked the ability to protect against LDL oxidation in a coculture model of the artery wall and, in fact, appeared to be proinflammatory (14). Our present results indicate that these proatherogenic effects of apoA-II can be reversed by overexpression of apoA-I. Whereas mAIItg mice exhibited increased atherosclerosis, combined hAItg/mAIItg mice exhibited about the same level of atherogenesis as nontransgenic mice (Fig. 4). These results are similar to the results of studies with human apoA-II transgenic mice (25). In parallel with the decrease in atherosclerosis, HDL from combined hAItg/mAIItg mice regained the capacity to protect against the proinflammatory effects of oxidized LDL as compared with HDL from mAIItg mice. These data support the concept that one of the protective effects of HDL with respect to atherosclerosis relates to its anti-inflammatory properties. Some studies have also suggested that apoA-II may influence reverse cholesterol transport (9, 36) and possibly influence interactions with the HDL scavenger receptor class B type I. These latter properties could also clearly influence the antiatherogenic properties of HDL. It is unclear why HDL enriched in apoA-II exhibits reduced anti-inflammatory properties (Fig. 5). Perhaps apoA-II influences the removal of oxidized lipids, although lipid hydroperoxide levels in apoA-II transgenic HDL as compared with wild-type mouse HDL are not discernibly different (14).

We conclude that apoA-II serves at least two functions. One is to modulate the structure and function of HDL by influencing lipolysis by HL, somewhat analogous to the role of apoC-III in VLDL lipolysis. Another is to modulate the anti-inflammatory/proinflammatory properties of HDL. Both of these functions depend in part on the ratio of apoA-I to apoA-II.

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REFERENCES

1. Tall, A. 1990. Plasma high density lipoproteins. Metabolism and relationship to atherogenesis. *J. Clin. Invest.* **86:** 379–384.
2. Karathanasis, S. K. 1992. Lipoprotein metabolism: high density lipoprotein. *In* Molecular Genetics of Coronary Artery Disease. A. J. Lusis, J. L. Rotter, and R. S. Sparkes, editors. S. Karger, Basel, Switzerland. 140–171.
3. Miller, N. E. 1987. Associations of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and atherosclerosis. *Am. Heart J.* **113:** 589–597.
4. Watson, A. D., M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, T. M. McIntyre, B. N. La Du, A. M. Fogelman, and J. A. Berliner. 1995. Effect of platelet-activating factor acetylhydrolase on formation and action of minimally oxidized LDL. *J. Clin. Invest.* **95:** 774–782.
5. Rubin, E. M., R. M. Krauss, E. A. Spangler, J. G. Verstuyft, and S. M. Clift. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature.* **353:** 265–267.
6. Rubin, E. M., B. Y. Ishida, S. M. Clift, and R. M. Krauss. 1991. Expression of human apolipoprotein AI in transgenic mice results in reduced plasma levels of murine apolipoprotein AI and the appearance of two new high density lipoprotein size subclasses. *Proc. Natl. Acad. Sci. USA.* **88:** 434–438.
7. Badimon, J. J., L. Badimon, and V. Fuster. 1990. Regression of atherosclerotic lesions by HDL plasma fraction in cholesterol-fed rabbits. *J. Clin. Invest.* **85:** 1234–1241.
8. Deeb, S. S., K. Takata, R. Peng, G. Kajiyama, and J. J. Albers. 1990. A splice-junction mutation responsible for familial apolipoprotein AII deficiency. *Am. J. Hum. Genet.* **46:** 822–827.
9. Barbaras, R., P. Puchois, J. C. Fruchart, and G. Ailhaud. 1987. Cholesterol efflux from cultured adipose cells is mediated by LpAI particles but not by LpAI:AII particles. *Biochem. Biophys. Res. Commun.* **142:** 63–69.
10. Zhong, S., I. J. Goldberg, C. Bruce, E. M. Rubin, J. L. Breslow, and A. Tall. 1994. Human AII inhibits hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and CETP in transgenic mice. *J. Clin. Invest.* **94:** 2457–2467.
11. Warden, C. H., C. C. Hedrick, J-H. Qiao, L. W. Castellani, and A. J. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein AII. *Science.* **261:** 469–472.
12. Hedrick, C. C., L. W. Castellani, C. H. Warden, and A. J. Lusis. 1993. Influence of mouse apolipoprotein AII on plasma lipoproteins in transgenic mice. *J. Biol. Chem.* **268:** 20676–20682.
13. Doolittle, M. H., R. C. LeBoeuf, C. H. Warden, L. M. Bee, and A. J. Lusis. 1990. A polymorphism affecting apolipoprotein A-II translational efficiency determines high density lipoprotein size and composition. *J. Biol. Chem.* **265:** 16380–16388.
14. Castellani, L. W., M. Navab, B. J. Van Lenten, C. C. Hedrick, S. Y. Hama, A. M. Gotoa, A. M. Fogelman, and A. J. Lusis. 1997. Overexpression of apolipoprotein AII in transgenic mice converts high density lipoproteins to proinflammatory particles. *J. Clin. Invest.* **100:** 464–474.
15. Homanics, G. E., H. V. DeSilva, J. Osada, S. H. Zhang, H. Wong, J. Borensztain, and N. Maeda. 1995. Mild dyslipidemia following targeted inactivation of the hepatic lipase gene. *J. Biol. Chem.* **270:** 2974–2980.
16. Qiao, J. H., P. Z. Xie, M. C. Fishbein, J. Kreuzer, T. A. Drake, L. L. Demer, and A. J. Lusis. 1994. Pathology of atheromatous lesions in inbred and genetically engineered mice. Genetic determination of arterial calcification. *Arterioscler. Thromb. Vasc. Biol.* **14:** 1480–1497.
17. Lusis, A. J., B. A. Taylor, D. Quon, S. Zollman, and R. C. LeBoeuf. 1987. Genetic factors controlling structure and expression of apolipoproteins B and E in mice. *J. Biol. Chem.* **262:** 7594–7604.
18. Nilsson-Ehle, P., and M. C. Schotz. 1976. A stable radioactive substrate emulsion for assay of lipoprotein lipase. *J. Lipid Res.* **17:** 536–541.
19. Peterson, J., G. Bengstsson-Olivecrona, and T. Olivecrona. 1986. Mouse preheparin plasma contains high levels of hepatic lipase with low affinity for heparin. *Biochim. Biophys. Acta.* **878:** 65–70.

20. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
21. Mowri, H-O., W. Patsch, L. C. Smith, A. M. Gotto, Jr., and J. R. Patsch. 1992. Different reactivities of high-density lipoprotein 2 subfractions with hepatic lipase. *J. Lipid Res.* **33:** 1269–1279.
22. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
23. Pawlowski, N. A., E. L. Abraham, S. Pointer, W. A. Scott, and Z. A. Cohen. 1985. Human monocyte:endothelial cell interaction in vitro. *Proc. Natl. Acad. Sci. USA.* **82:** 8208–8212.
24. Fogelman, A. M., K. Sykes, B. J. Van Lenten, M. C. Territo, and J. A. Berliner. 1988. Modification of the Recalde method for the isolation of human monocytes. *J. Lipid Res.* **29:** 1243–1247.
25. Escola-Gil, J. C., A. Marzal Casacuberta, J. Julve-Gil, B. Y. Ishida, J. Ordonez-Llanos, L. Chan, F. Gonzalez-Sastre, and F. Blanco-Vaca. 1998. Human apolipoprotein AII 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.* **39:** 457–462.
26. Shih, D. M., L. Gu, Y. R. Xia, M. Navab, W. F. Li, S. Y. Hama, L. W. Castellani, C. E. Furlong, L. G. Costa, A. M. Fogelman, and A. J. Lusis. 1998. Mice lacking serum paraoxonase are susceptible to organophophate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
27. Castro, G., L. P. Nihoul, C. Dengremont, C. de Geitere, B. Delfly, A. Tailleux, C. Fievet, N. Duverger, P. Denefle, and J. C. Fruchart. 1997. Cholesterol efflux, lecithin-cholesterol acyltransferase activity, and pre-beta particle formation by serum from human apolipoprotein. A-I and apolipoprotein A-I/apolipoprotein A-II transgenic mice consistent with the latter being less effective for reverse cholesterol transport. *Biochemistry.* **36:** 2243–2249.
28. Dansky, H. M., S. A. Charlton, C. B. Barlow, M. Tamminen, J. D. Smith, J. S. Frank, and J. L. Breslow. 1999. ApoAI inhibits foam cell formation in apoE-deficient mice after monocyte adherence to endothelium. *J. Clin. Invest.* **104:** 31–39.
29. Mehrabian, M., J. H. Qiao, R. Hyman, D. Ruddle, C. Laughton, and A. J. Lusis. 1993. Influence of the apoAII gene locus on HDL levels and fatty streak development in mice. *Arterioscler. Thromb. Vasc. Biol.* **13:** 1–10.
30. Purcell-Huynh, D. A., A. Weinreb, L. W. Castellani, M. Mehrabian, M. H. Doolittle, and A. J. Lusis. 1995. Genetic factors in lipoprotein metabolism. Analysis of a genetic cross between inbred mouse strians NZB/BINJ and SM/J using a complete linkage map approach. *J. Clin. Invest.* **96:** 1845–1858.
31. Weng, W., N. A. Brandenburg, S. Zhong, J. Halkias, L. Wu, X. C. Jiang, A. Tall, and J. L. Breslow. 1999. ApoAII maintains HDL levels in part by inhibition of hepatic lipase. Studies in apoAII and hepatic lipase knockout mice. *J. Lipid Res.* **40:** 1064–1070.
32. Weng, W., and J. L. Breslow. 1996. Dramatically decreased high density lipoprotein cholesterol, increased remnant clearance, and insulin hypersensitivity in apolipoprotein AII knockout mice suggest a complex role for apolipoprotein AII in atherosclerosis susceptibility. *Proc. Natl. Acad. Sci. USA.* **93:** 14788–14794.
33. Magill, P., S. N. Rao, N. E. Miller, A. Nicoll, J. Brunzell, J. St Hilaire, and B. Lewis. 1982. Relationships between the metabolism of high-density and very-low-density lipoporteins in man; studies of apolipoprotein kinetics and adipose tissue lipoprotein lipase activity. *Eur. J. Clin. Invest.* **12:** 113–120.
34. Blades, B., G. L. Vega, and S. M. Grundy. 1993. Activities of lipoprotein lipase and hepatic triglyceride lipase in post heparin plasma of patients with low concentrations of HDL cholesterol. *Arterioscler. Thromb.* **13:** 1227–1235.
35. Boisfer, E., G. Lambert, V. Atger, N. Q. Tran, D. Pastier, C. Benetollo, J. F. Trottier, I. Beaucamps, M. Antonucci, M. Lapland, S. Griglio, J. Chambaz, and A. D. Kalopissis. 1999. Overexpression of human apolipoprotein AII in mice induce hypertriglyceridemia due to defective very low density lipoprotein hydrolysis. *J. Biol. Chem.* **274:** 11564–11572.
36. Hime, N. J., P. H. Barter, and K. A. Rye. 1998. The influence of apolipoproteins on the hepatic-lipase-mediated hydrolysis of HDL phospholipid and triaclyglycerol. *J. Biol. Chem.* **273:** 27191–27198.
37. DeOliveria, E., M. Silva Kong, Z. Han, C. Starr, E. M. Kass, S. H. Juo, D. Foster, H. M. Dansky, M. Merkel, K. Cundey, E. A. Brinton, J. L. Breslow, and J. D. Smith. 1999. Metabolic and genetic determinants of HDL metabolism and hepatic lipase activity in normolipidemic females. *J. Lipid Res.* **40:** 1211–1221.
38. Warden, C. H., A. Daluski, X. Bu, D. A. Purcell-Huynh, C. DeMeester, B. H. Shieh, D. L. Puppione, R. M. Gray, G. M. Reaven, Y. D. Chen, and A. J. Lusis. 1993. Evidence for linkage of the apolipoprotein AII locus to plasma apolipoprotein AII and FFA levels in mice and humans. *Proc. Natl. Acad. Sci. USA.* **90:** 10886–10890.
39. Rajput-Williams, J., J. Eyre, M. N. Nanjee, D. Crook, J. Scott, N. E. Miller, I. Baker, and S. E. Humphries. 1989. Plasma lipoprotein lipids in relation to the MspI polymorphism of the apolipoprotein AII gene in Caucasian men. *Atherosclerosis.* **77:** 31–36.
40. Kessling, A. M., J. Rajput-Williams, D. Bainton, J. Scott, N. E. Miller, I. Baker, and S. E. Humphries. 1988. DNA polymorphisms of the apoAII and AI-CIII-AIV genes: a study in men selected for difference in HDL-C concentration. *Am. J. Hum. Genet.* **42:** 458–467.