Polymorphism in the human apolipoprotein A-I gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro.

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J Clin Invest. 1992;89(6):1796-1800. https://doi.org/10.1172/JCI115783.

Research Article

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Polymorphism in the Human Apolipoprotein A-I Gene Promoter Region

Association of the Minor Allele with Decreased Production Rate In Vivo and Promoter Activity In Vitro

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Abstract

We investigated a common polymorphism in the human apolipoprotein A-I gene promoter at a position 76 bp upstream of the transcriptional start site. 54 human subjects, whose apoAI production rates had been determined by apoAI turnover studies, were genotyped at this polymorphic position by a novel technique using polymerase chain reaction followed by primer extension. 35 subjects were homozygous for a guanosine (G) at this locus and 19 were heterozygous with a guanosine and adenosine (A). The apoAI production rates were significantly lower (by 11%) in the G/A heterozygotes than in the G homozygotes ($P = 0.025$). In spite of the apparent effect of this apoAI gene promoter polymorphism on the apoAI production rate, there was no effect on HDL cholesterol or apoAI levels. To investigate whether the observed difference in apoAI production rates was related to differential gene expression of the two alleles, promoters containing either allele were linked to the reporter gene chloramphenicol acetyltransferase, and relative promoter efficiencies were determined after transfection into the human HepG2 hepatoma cell line. The A allele expressed only 68%±5% as well as the G allele, a result consistent with the in vivo apoAI production rate data. (*J. Clin. Invest.* 1992. 89:1796–1800.) Key words: apolipoprotein A-I turnover • gene expression • high density lipoprotein • polymerase chain reaction • transfection

Introduction

Apolipoprotein A-I is the major protein constituent of high density lipoproteins, and plasma levels of apoAI and HDL cholesterol are inversely correlated with incidence of coronary artery disease (1). ApoAI and HDL cholesterol levels may be controlled by the apoAI production rate (AIPR),[1] which includes apoAI synthesis, intracellular transport, secretion, and/or apoAI plasma clearance, expressed as the fractional catabolic rate (FCR). Previous studies from this laboratory have shown that interindividual differences in plasma apoAI and HDL cholesterol levels of subjects on a fixed diet are inversely correlated with apoAI FCR, but not correlated with AIPR (2, 3). However, in certain situations AIPR can play an important role in determining plasma apoAI and HDL cholesterol levels. Interindividual differences in dietary response of apoAI and HDL cholesterol levels correlate with differences in response of AIPR (4). In addition, apoAI gene lesions precluding apoAI synthesis have been associated with very low HDL cholesterol levels (5). Furthermore, apoAI gene overexpression in transgenic mice causes increased AIPR, and elevates apoAI and HDL cholesterol levels (6).

The region immediately 5′ to the apoAI gene, the apoAI promoter, is required for apoAI gene expression. Human apoAI genomic fragments which contain a promoter consisting of only 256 bp upstream of the apoAI transcriptional start site are sufficient for expression in liver, but not in intestine, in transgenic mice (5). Four protein-binding domains have been mapped in this proximal promoter region between −228 bp and +17 bp by DNase I footprinting experiments (7). A common polymorphism exists in the human apoAI promoter region which was originally detected by DNA sequencing of genomic clones (8, 9). This polymorphism consists of either the more common guanosine (G), or less commonly an adenosine (A) at a position 76 bp upstream of the transcriptional start site (10). This site is on the boundary of one of the footprints (7) and is also within an inverted repeat of 14 out of 15 bp (10). In the current study we compared AIPR of human subjects who had been genotyped at the −76 bp polymorphic locus by a novel method using the techniques of polymerase chain reaction (PCR) followed by primer extension. We found that the minor A allele was associated with lower AIPR in vivo. To test whether the G to A sequence change could directly affect AIPR, we measured promoter activity of the two alleles in vitro by linkage to a chloramphenicol acetyltransferase (CAT) reporter gene and transfection into the HepG2 human hepatoma cell line. The minor A allele had only 68% of the promoter activity of the major G allele, and therefore this difference in promoter efficiency could account for the decrease in the in vivo AIPR observed in the G/A heterozygotes.

Methods

ApoAI production rate determinations. ApoAI turnover studies were done on 54 volunteers with a wide range of HDL cholesterol levels residing as inpatients at the Rockefeller University Hospital by methods described previously in detail (2–4). These volunteers consisted of both males and females who were either healthy or patients from the lipid clinic. None of the subjects were receiving estrogen therapy or any other medication known to alter AIPR. All studies were performed while the subjects were fed whole food metabolic diets, with fat accounting for 42% of the calories (2). HDL cholesterol levels were carefully determined by averaging the results from five separate blood drawings made during the final two weeks of the four-week metabolic diet. ApoAI FCR was determined experimentally by the Matthews method,

Portions of this work were presented in abstract form at the American Heart Association Scientific Sessions, 11–14 November 1991, Anaheim, CA.

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Received for publication 23 August 1991 and in revised form 5 February 1991.

1. *Abbreviations used in this paper:* A, adenosine; AIPR, apoAI production rate; CAT, chloramphenicol acetyltransferase; FCR, fractional catabolic rate; G, guanosine; PCR, polymerase chain reaction.

J. Clin. Invest.

0021-9738/92/06/1796/05 $2.00
Volume 89, June 1992, 1796–1800

following the plasma die-away curves after bolus injection of [^{125}I]apoAI (2). The apoAI production rate, also known as transport rate, was calculated by the following equation (2):

$$\text{AIPR} = \frac{\text{FCR} \times \text{apoAI concentration} \times \text{plasma vol}}{\text{body wt}}$$

ApoAI genotyping. DNA from the subjects was prepared from 0.5 ml of frozen buffy coat samples using the serum separation tube method as described (11). Amplification of the apoAI promoter region was accomplished by PCR with the primers CCCCACCCGGGAGACCTGCAAGCC (−270 to −247 bp relative to the transcription start site) and CTCTAAGCAGCCAGCTCTTGCAGGGCCT (+1 to −27 bp). PCR reactions were carried out with 5 μl of DNA under oil in a final vol of 50 μl in a solution containing 10% DMSO, 16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH8.8), 6.7 mM magnesium chloride, 10 mM DTT, 170 μg/ml BSA, 0.2 mM each deoxynucleotide triphosphate, 25 pmol of each primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Samples were heated to 95° for 1 min and subjected to 30 cycles at 93°C for 1 min, 70°C for 1 min, and 72°C for 2 min. 5 μl was analyzed on a 1.3% agarose gel to check for the presence of the 271-bp amplification product. The rest of the sample was purified away from the primers and unincorporated nucleotides using a Gene Clean II kit according to the manufacturer's specifications (Bio 101, Inc., Vista, CA). The purified PCR product was genotyped at the −76 position by a primer extension assay. The primer GGACCAGTGAGCAGCAACA (−102 to −84 nt) was labeled on its 5′ end with T4 polynucleotide kinase and [^{32}P]ATP. The 10-μl primer extension reaction contained 4 μl of the purified PCR product, 16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH8.8), 6.7 mM magnesium chloride, 10 mM DTT, 200 μg/ml BSA, 50 μM each of dGTP, dCTP, dTTP, 200 μM dideoxyATP, 5,000 cpm of labeled primer, and 2.5 U of Taq polymerase. The extension was allowed to proceed for 10 cycles of 93°C for 1 min, 45°C for 1 min, and 70°C for 1 min. The primer extension products are denatured by heating at 90°C after addition of 4 μl of an 80% formamide sequencing dye buffer, and loaded onto a 1.5 mm thick, 15 cm long, 12% polyacrylamide, 7 M urea gel. The gel was run at 25 mA for 1 h, dried, and exposed to x-ray film.

In vitro expression studies. The human apoAI genomic clone previously reported by this laboratory contains a G at −76 bp (8). To obtain the A allele for comparison with the G allele in expression studies, we inserted a 321-bp SmaI/StuI restriction fragment (−253 to +68 bp) into the pSelect vector using HindIII and BamHI linkers, and made an oligonucleotide directed mutation of the −76 G to an A using an Altered Sites kit according to the manufacturer's specification (Promega Biotec, Madison, WI). The G and A alleles were confirmed by DNA sequencing, and both promoters were subcloned into the pKT vector (12) so that they were in the proper orientation to drive the CAT gene. Transfections of HepG2 cells, beta galactosidase assays, and CAT assays were performed as described previously (13). CAT expression vectors were cotransfected with pCH110 (14), a beta-galactosidase expression vector used to normalize for transfection efficiency. In each experiment duplicate plates were transfected for each allele and extracts with equal amounts of beta-galactosidase activity were assayed for CAT activity.

Statistics. Values given in the text are means±standard deviation. Unpaired *t* tests, chi square, and Fisher's exact test were performed using the InStat statistical software program from GraphPAD Software (San Diego, CA). All *t* tests are two-tailed unless otherwise noted.

Results

To determine the 54 subjects' genotypes at the polymorphic site in the apoAI promoter we developed a novel primer extension assay shown schematically in Fig. 1 *A*. First, the subjects' apoAI promoter region was amplified by PCR to yield a 271-bp fragment. The amplified fragment was annealed to a 5′ end-labeled sense strand 19-mer primer which ended 6 bases upstream of the polymorphic base. This primer was extended using Taq polymerase in the presence of dGTP, dCTP, dTTP, and dideoxyATP. Since there is no A between the primer and the polymorphic position, if an A is present at position −76 the primer will extend to a 25 mer; however, if a G is present at the polymorphic position, the primer will be extended to the next A at position −64 yielding a 37-mer product. If an individual is heterozygous at this position, both extension products should be present in equal amounts. This novel primer extension assay was easy to perform, reproducible, and yielded unambiguous results; and furthermore, this type of assay can be used to genotype most single base polymorphisms in place of the allele-specific oligonucleotide hybridization method, which requires carefully controlled hybridization and washing conditions. An autoradiograph of a denaturing gel from a primer extension assay is shown in Fig. 1 *B*. 35 subjects were homozygous for the G allele and 19 were G/A heterozygotes. The calculated frequencies of the major G allele and the minor A allele were 82.4% and 17.6%, respectively. We detected no A homozygotes within our sample; based upon the heterozygote frequency the expected frequency of A homozygotes was 3.1%, or 1.7 subjects out of our sample of 54. The gene frequencies determined were not significantly different than expected according to the Hardy Weinberg Law ($P = 0.29$, chi square analysis).

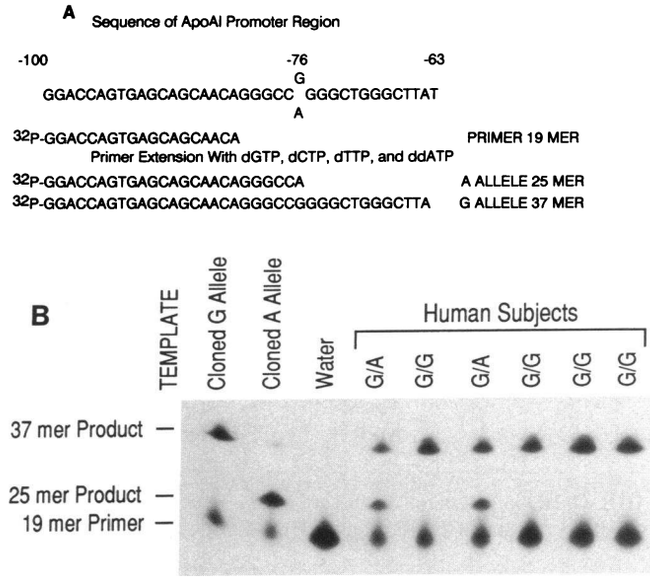

Figure 1. Primer extension assay. (*A*) The sense strand of the polymorphic region is shown along with an end-labeled 19-mer primer. Primer extension is performed in the presence of dideoxyATP, along with the other three deoxynucleotide triphosphates. If an A is present at the polymorphic position the extension is terminated there yielding a 25-mer product. If a G is present at the polymorphic position the extension is terminated at the first A another 12 nucleotides beyond the polymorphic position, yielding a 37-mer product. (*B*) Example of the primer extension assay performed on cloned G or A alleles, and PCR products derived from water, as a control, and DNA from six human subjects.

Plasma apoAI and HDL cholesterol levels, as well as AIPR and FCR, were determined in the 54 subjects as described above. AIPR from males and females and their genotypes at the −76 position in the apoAI promoter are shown in Table I. There was no significant difference in the AIPR between males

Table I. HDL-Cholesterol, ApoAI, and ApoAI Production Rate by Sex and Genotype

Genotype			Females	Males	Total
	n		18	17	35
G/G	HDL-C	*mg/dl*	55.2 (23.2)	44.2 (12.8)	49.9 (19.4)
	ApoAI	*mg/dl*	151.1 (34.0)	126.4 (28.4)	137.8 (33.6)
	AIPR	*mg/kg per d*	12.46 (1.35)*	12.27 (2.73)	12.37 (2.10)‡
			12	7	19
G/A	HDL-C	*mg/dl*	59.0 (19.1)	44.3 (20.8)	52.8 (20.7)
	ApoAI	*mg/dl*	145.3 (28.3)	124.8 (31.7)	137.7 (30.5)
	AIPR	*mg/kg per d*	11.23 (1.63)*	10.77 (2.13)	11.06 (1.78)‡
			30	24	54
Total	HDL-C	*mg/dl*	56.7 (21.4)§	44.2 (15.1)§	51.2 (19.7)
	ApoAI	*mg/dl*	148.8 (31.5)‖	125.9 (28.7)‖	137.8 (32.2)
	AIPR	*mg/kg per d*	11.97 (1.57)	11.84 (2.61)	11.91 (1.88)

HDL-C, HDL cholesterol. Values are mean (± standard deviation). * Female G/G vs. female G/A, $P = 0.032$. ‡ Total G/G vs. total G/A, $P = 0.025$. § Female HDL-C vs. male HDL-C, $P = 0.019$. ‖ Females ApoAI vs. male ApoAI. $P = 0.008$.

and females. The 30 females had apoAI production rates ranging from 8.54 to 14.23 mg/kg per d, with a mean of 11.97±1.57 mg/kg per d. The 24 males had apoAI production rates ranging from 7.04 to 18.40 mg/kg per d, with a mean of 11.84±2.62 mg/kg per d. However, for both sexes the G/A heterozygotes had a lower AIPR than did the G homozygotes; a 12% decrease was observed in the G/A males, and a 10% decrease in the G/A females. This difference was significant for the females alone and for the males and females pooled. This lack of significance in the males alone was due to a smaller sample size with more variation than was observed in the females. However, pooling the AIPR data of both sexes led to a greater level of statistical significance than was evident in the females alone (pooled $P = 0.025$, female $P = 0.032$). For the pooled data, the G homozygotes had apoAI production rates of 12.37±2.10 mg/kg per d, while it was 11.06±1.78 mg/kg per d for the heterozygotes. This represents an 11% mean decrease in AIPR of the G/A heterozygotes compared to the G homozygotes. Each subject's individual AIPR and the mean difference in AIPR between the G homozygotes and the G/A heterozygotes are shown graphically in Fig. 2.

As shown in Table I, females had significantly higher levels of plasma apoAI ($P = 0.008$) and HDL cholesterol ($P = 0.019$) than males, but within each sex, or combined, there was no significant difference in apoAI or HDL cholesterol levels between the G homozygotes and the G/A heterozygotes. ApoAI FCR was also compared in this population. The inverse correlation between HDL cholesterol levels and apoAI FCR in all of the subjects was −0.81 ($P < 0.001$) (Brinton, E. A., S. Eisenberg, and J. L. Breslow, manuscript in preparation). For all males apoAI FCR was .274±.066 pools/d, while it was .248±.077 pools/d for all females. The trend of higher apoAI FCR in males corresponds to lower apoAI and HDL cholesterol levels, although the increase in apoAI FCR in males compared to females failed to reach significance in this population ($P = 0.096$ by a one-tailed t test). There was no trend or significant difference in apoAI FCR between the −76 apoAI genotypes in either males or females alone, or pooled.

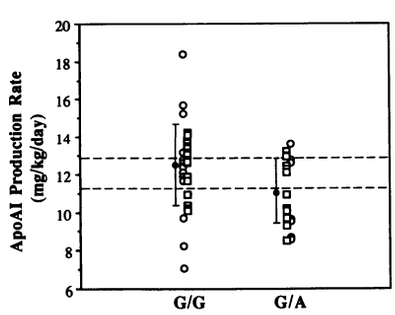

Figure 2. ApoAI production rate in G homozygotes and G/A heterozygotes. Individual AIPRs are shown for males (○) and females (□), along with the mean and standard deviation for each genotype. The dotted lines divide all the subjects into tertiles of AIPR.

The overall heterozygote frequency in our sample was 35.2%; however, when we divided the subjects into tertiles of AIPR, we found the heterozygote frequency to be 22.2% in the highest tertile, 27.8% in the middle tertile, and 55.5% in the lowest tertile (Fig. 2). The increase in heterozygotes observed in the bottom AIPR tertile was significantly different from the number of heterozygotes in the top tertile, as was the difference observed between the bottom tertile and the pooled top plus middle tertiles of AIPR ($P < 0.05$, Fisher's exact test, one-tailed analysis).

To address whether the observed difference in apoAI production rates of the G homozygotes versus the G/A heterozygotes was based upon any inherent difference in promoter efficiency between the two alleles, we tested both alleles by fusion with a CAT reporter gene and transfection into HepG2 cells. We chose to use HepG2 cells in these experiments since they express their endogenous apoAI gene and are readily transfected. Fig. 3 shows the results of a typical CAT assay: in this experiment the mean conversion of chloramphenicol was 39.5% for the G allele promoter construction and 24.6% for the A allele construction. In four separate experiments shown in Table II, each with duplicates within 15% of each other, the less common A allele expressed only 68%±5% as well as the more common G allele ($P < 0.0001$). This 32% reduction in the efficiency of the −76 A promoter corresponds qualitatively to that expected based upon the apoAI production rate data.

Discussion

We have demonstrated that a relatively common G to A transition within the proximal promoter region of the human apoAI

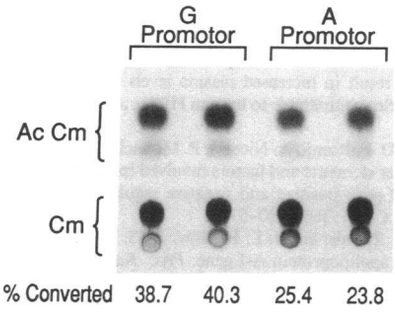

Figure 3. Promotor activity of G and A alleles. 325 basepair promoters with either a G or A at the polymorphic position were linked to a CAT reporter gene as described in Methods. HepG2 cells were transfected with 10 μg of plasmid DNA along with 10 μg of a beta-galactosidase expression vector. Extracts containing equal amounts of beta-galactosidase activity were assayed for CAT activity. Acetylated chloramphenicol (*Ac Cm*) was separated from chloramphenicol (*Cm*) by thin layer chromatography. The percentage of chloramphenicol converted to the acetylated forms was determined by cutting the chromatogram followed by liquid scintillation counting.

gene is associated with decreased in vivo AIPR in both males and females as determined by turnover studies of radioiodinated apoAI in 54 subjects fed a metabolic diet. This difference was demonstrated both by an 11% reduction in mean AIPR in the G/A versus the G/G subjects, and by a higher frequency of G/A subjects in the lowest tertile of AIPR. Although it is possible that this effect is not due to the −76 polymorphism, but instead due to some other genetic change in which it is in linkage disequilibrium, a molecular mechanism responsible for this in vivo difference is suggested by the finding of significantly lower promoter activity of the −76 A allele in an in vitro transfection assay. In this in vitro assay the effects must be due to the −76 polymorphism since the two expression plasmids were identical with the exception of the −76 position. Although the average AIPR of the G/A heterozygotes was only 11% less than of the G homozygotes, this is not far removed from the expected difference based upon the promoter activity of the cloned alleles. The A allele promoter expression was 32% less than that of G allele promoter; therefore, the decrease in overall expression predicted upon going from two G alleles to one G and one A allele is 16% (half of 32%). Since other transcriptional and nontranscriptional factors may influence AIPR, it is reasonable to expect that the observed decrease in the in vivo AIPR might be less pronounced than the reduction of transcription of the isolated promoter. Thus our data suggest that a common substitution of A for G in the apoAI gene promoter can cause decreased apoAI gene expression and protein production.

We speculate that there are at least two possible mechanisms which could account for the lower level of expression of the A allele compared to the G allele. The A allele may have an altered affinity for *trans*-acting factor(s) binding to this region of the proximal promoter. Although the −76 position of the apoAI promoter is not included in footprint B of Papazafiri et al. (7), which extends upstream from −77, the resolution of this type of assay is not precise enough to rule out direct binding to the −76 bp. An alternate mechanism is that the presence of the −76 A, which forms a more perfect inverted repeat in the promoter, may allow for the formation of DNA secondary structure which might interfere with protein–protein interactions of the transcriptional apparatus.

A species-specific difference in plasma apoAI levels in two nonhuman primates has been correlated with a twofold difference in apoAI gene transcription rate measured in isolated nuclei (15). When the apoAI promoter activities of these two primates were compared by transient transfection, a 1.4–3.0-fold higher expression level was observed from the primate with the higher apoAI transcription rate (15). There were 15 nucleotide differences in the 494-bp promoter region from −231 to +263 bp in these two species (15). Although the specific nucleotide change(s) leading to the difference in promoter efficiency was not determined, both species contained a G, similar to the more common human allele, at the base corresponding to the −76 position in the human apoAI promoter. In this case of interspecies comparison, the twofold difference in apoAI transcription rate was associated with a difference in plasma levels of apoAI. The minor difference in promoter efficiency of the two human alleles that are the subject of this study was reflected in a small, but significant, difference in AIPR. However, this small effect on AIPR was not associated with any difference in plasma HDL cholesterol or apoAI levels. In our human subjects an even more overriding effect on plasma apoAI and HDL cholesterol levels was found. Within this population, plasma HDL cholesterol and apoAI levels are correlated with apoAI FCR, but not with AIPR (2, 3).

Our results contrast those of Pagani et al. who reported an increase in the frequency of the A allele in females, but not males, who are in the top decile of HDL cholesterol (10). We cannot explain this discrepancy, except to note that HDL cholesterol levels that we report here were the average of five separate determinations from subjects on a metabolic diet, thereby minimizing the influence of diet and assay variability. The Pagani study did not measure AIPR in vivo or gene expression (10).

To our knowledge, the apoAI gene promoter polymorphism at position −76 bp is the first example of an apolipoprotein gene regulatory mutation. It also provides an interesting example of how a common genetic variation causing a mild change in gene function can influence a related physiological parameter in vivo.

Table II. Normalized CAT Activity for −76 G and A ApoAI Promoter Constructions

	G Allele	A Allele
	%	%
Experiment 1	101.94	64.19
	98.06	60.21
Experiment 2	98.85	70.39
	101.15	75.29
Experiment 3	99.69	67.21
	100.31	69.29
Experiment 4	92.62	66.46
	107.38	73.98
Mean (SD)	100 (4)	68 (5)

Within each experiment CAT activity (percentage of acetylated chloramphenicol) for each transfection was normalized to the mean of the two −76 G construction transfections. Transfections in experiments 1 and 3 were performed with one pair of plasmid preparations, and transfections in experiments 2 and 4 were performed with a separate pair of plasmid preparations. Mean expression levels of G and A CAT constructions are significantly different by a two-tailed *t* test ($P < 0.0001$).

Acknowledgments

The authors would like to thank Mr. Yuying Zhu for excellent technical assistance.

This work is sponsored by grants from the National Institutes of Health (HL-32435, HL-33714, and HL-02034), a General Clinical Research Center grant (RR-00102), a Clinical Nutrition Research Unit grant (CA-29502), a New York Heart Association Grant-In-Aid, and a grant from the Bankers Trust Company Group. Jonathan Smith is supported by an Investigatorship Award from the New York Heart Association.

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