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The Relation of 5'flanking Region of the Apolipoprotein(a) Gene to Serum Lipoprotein(a) Level

Hiroshi Shimizu¹, Takahiro Taniguchi¹, Yoshio Fujioka², Akihiro Takahashi¹, Yuichi Ishikawa², and Mitsuhiro Yokoyama¹

Lipoprotein(a) (Lp(a)) is an atherogenic lipoprotein whose serum concentration is considered to be mainly determined by apolipoprotein(a) (apo(a)) size and its production rate. Three types of variation have been identified in the apo(a) gene; a size polymorphism in the coding region, a pentanucleotide repeat polymorphism in the promoter region, and sequence variation in coding and non-coding regions of the gene including a C/T polymorphism at +93 from the transcription start. To clarify if the analysis of these polymorphisms could predict the serum levels of Lp(a), we subcloned the 1.4 kb 5'flanking region of apo(a) gene from subjects with low (<10 mg/dl, n=2) and high (>100 mg/dl, n=3) serum levels of Lp(a) and compared the promoter activity by in vitro transcription assay with HepG2 cells and furthermore studied the response of interleukin-6 and estradiol which had been reported to alter serum levels of Lp(a). Every subject in our study showed C at +93. The basal promoter activity of high Lp(a) group was not higher than that of low Lp(a) group. We did not find that a size polymorphism and pentanucleotide repeat polymorphism in this region affected the promoter activity. Interleukin-6 had a tendency to enhance the promoter activity, but estradiol did not. In conclusion, serum level of Lp(a) in individual was not predicted by the analysis of polymorphisms of 1.4 kb 5'flanking region of apo(a) gene. There may be other factors that are responsible for the regulation of the concentration of serum levels of Lp(a).

Key Words: Lipoprotein(a), Apolipoprotein(a), 5'flanking Region, Polymorphism.

Introduction

The First Department of Internal Medicine¹, and The Faculty of Health Sciences², Kobe University School of Medicine, Kobe, and The Department of Internal Medicine², Cardiovascular Division, Hyogo College of Medicine, Nishinomiya, Japan.

Lipoprotein(a) (Lp(a)) is an atherogenic lipoprotein comprised of a low density lipoprotein (LDL) like particle and a single large glycoprotein called apolipoprotein(a) (apo(a)), linked by a disulfide bond to apolipoprotein B-
Lp(a) formation can occur in plasma through the association of apo (a) with circulating LDL. Serum concentrations of Lp(a) vary over a wide range among individuals, but are remarkably stable in any given individuals. The differences in Lp(a) production rate affect the serum levels of Lp (a), even among the individuals with the same apo(a) isoform. It was reported that the apo(a) gene transcription is mainly regulated by hepatocyte nuclear factor-1α. As for the mechanism responsible for the difference in Lp(a) production, some family studies suggested that plasma concentrations of Lp(a) are virtually entirely heritable and that the apo(a) gene accounts for almost the whole heritability.

Some genetic variation associated with plasma Lp(a) levels have been identified in the apo (a) gene. First, the inverse relationship between the size of apo(a) protein and the concentration of plasma Lp(a) have been reported. Apo(a) isoforms are of different molecular size because of a size polymorphism in the coding region (kringle IV type 2 variable number of tandem repeats (VNTR)). Second, Wade et al. reported the 1.4 kb sequence of the 5' flanking region of the apo(a) gene and they proposed that this promoter activity of a subject of relatively high plasma Lp(a) concentration was greater than that of a subject of low Lp(a) level. This 5'flanking region of the apo(a) gene has TTTTA pentanucleotides repeat in most upstream region, and the number of this pentanucleotides is thought to be associated with the serum Lp(a) concentration. The third is the sequence variation in coding and non-coding regions of the gene. For example, Zysow et al. reported that a C/T substitution at +93 position from the transcription start site reduced the transcription rate in transient transfection assay. Mancini et al. reported a DraIII restriction site polymorphisms in the kringle IV type 2 VNTR. Suzuki et al proposed that the apo(a) gene could be subclassified into four allelic types by polymorphism in the 5'flanking region, and mentioned the importance of the C/T substitution at +93 position. On the other hand, recent study showed no relation between these polymorphisms and the concentration of plasma Lp(a). The apo(a) gene was estimated to be responsible for 91% of the variance of plasma Lp(a) concentration and the number of kringle IV type 2 VNTR in the apo(a) gene is considered to account for 69% of the variation. However, Gaw et al. have suggested that the effect of the kringle IV VNTR on Lp(a) has been overestimated in previous studies and that the polymorphism explains only 9-10% of the variance in Lp(a) concentrations. Recent review showed that these polymorphisms can explain only about a half of the variance in Lp(a) concentrations in white populations and lesser in black Africans.

It is known that the plasma Lp(a) concentration increases in inflammation, which is related with interleukin 6 (IL-6) production, but the increased level is variable in individuals.
There are multiple sites of IL-6 responsive element in reported 5'flanking region of the apo(a) gene\(^{19}\). Estrogen therapy reduces the plasma Lp(a) level\(^{28,29}\). However, it has not been clarified whether these effects are different in the variance of apo(a) gene polymorphisms.

Now we investigated the question as to whether these polymorphisms of apo(a) gene correspond with plasma concentrations of Lp(a) in individuals, and examined the response of the promoter activities of apo(a) gene to IL-6 and estrogen. These findings suggest the existence of other factors to regulate the apo(a) gene transcription and production of apo(a) protein.

**Subjects and Methods**

**Materials**

Restriction enzymes (EcoRI, KpnI, BglII), GeneAmp\(^{\text{TM}}\) PCR reagent Kit with AmpliTaq\(^{\text{TM}}\) DNA polymerase for PCR, and DNA Ligation Kit were purchased from TAKARA Biomedicals (Kyoto, Japan). Primers for PCR were from Japan Bio Service (Saitama, Japan) and primers for DNA sequencing were from IWAKI GLASS (Chiba, Japan). GENECLEAN kit for DNA isolation were from BIO 101 (Vista, CA, USA). PicaGene\(^{\text{TM}}\) basic vector and luciferase assay kit were from TOYO INK MFG. CO. (Tokyo, Japan). Calcium Phosphate Transfection Kit was purchased from 5Prime\rightarrow 3Prime, Inc (Boulder, CO, USA). Sequenase\(^{\circledR}\) 7-deaza-dGTP Sequencing Kit for DNA sequencing was from USB (Cleveland, OH, USA). Lp(a) phenotyping kit was obtained from Sanwa Chemical Labo. (Nagoya, Japan). Other materials and chemicals were obtained from commercial sources.

**Subjects**

Five men with high or low Lp(a) level were selected. Three of them (designated H1, H2, and H3) had high level of serum Lp(a)(>100 mg/dl) and two (designated L1 and L2) had low level(≤10 mg/dl). H2 and H3 were diagnosed ischemic heart disease by coronary arteriography. There were not found diabetes mellitus, liver or kidney dysfunction, and other metabolic disorders except for hyperlipidemia in all subjects. They have never taken lipid-lowering and hormonal drug. We obtained blood samples from them in 12 h fasting condition. The protocol was approved by our hospital ethics committee, and all patients gave informed consent.

**Apo(a) phenotyping**

Apo(a) phenotypes of individuals were determined by SDS-PAGE and western blotting using Lp(a) phenotyping kit according to the F, B, S1, S2, S3, S4 (and their heterozygous types) nomenclature of Utermann et al.\(^{11}\). Recent powerful separation techniques and the increase in the sensitivity of the blotting procedure has increased the number of apo(a) isoforms to more than 38\(^{16}\), but we performed the phenotyping simply to examine the tendency of inverse relationship between the size of apo(a) protein and...
the concentration of serum Lp(a) and compare our results with data previously published.

**Determination of serum lipids and Lp(a) levels**

Serum cholesterol and triglyceride levels were determined by enzymatic methods. High density lipoprotein (HDL) cholesterol concentration was determined by Ca²⁺-heparin precipitation method. Serum levels of Lp(a) were determined using a TintElize immunoassay kit (Biopool AB, Umeå, Sweden).

**Genomic DNA extraction**

Genomic DNA of individual subjects was extracted from lymphocytes. Briefly, blood was obtained with heparin and lymphocytes were collected with addition of 0.2% NaCl and centrifugation. After cell lysis with 0.05 M Tris/HCl (pH 7.4), 0.1 M EDTA, 0.1 M NaCl, and 1.0% SDS, DNA was obtained with proteinase K treatment and phenol/isoamyl alcohol extraction.

**PCR and luciferase reporter gene construct**

Genomic DNA was digested by EcoRI and applied to PCR using Taq DNA polymerase. The set of primers used for PCR were the following: a1 (GGG GTACCGAATTCATTTGCGGAAAGATTGTATG) and a2 (GAAGATCTGCCAGTGCCCAGAAAGTGTGT). These probes were determined according to the sequence reported by Wade et al. Primer a2 was determined by the region from-34 to-11. PCR was performed in 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C in 30 cycles. The resulting fragments were isolated from agarose gels with GENE CLEAN. These products were digested with Kpn I and BglII and inserted into KpnI-BglII-digested PicaGene™ basic vector (PGV-B), promoterless luciferase reporter gene vector, to construct pAPO(a) vector.

**Cells**

HepG2 cells, human hepatoblastoma cell line, were kindly gifted from Dr. Allen D. Cooper (Stanford University) and maintained in modified Eagle’s minimum essential medium (MEM) supplemented with phenol red, 10% FBS, 0.3 mg/ml L-glutamate, 100 units/ml penicillin G, and 100 μg/ml streptomycin (medium A) at 37°C in humidified atmosphere.

**Transient transfection and assay of reporter gene constructs**

Plasmids for transfection were purified by Qiagen columns (Hilden, Germany). For transient transfection with constructed plasmids pAPO(a), HepG2 cells were seeded at 3x10⁵ cells per 60 mm dish. After 24 h, transfection were performed. For each dish, 10 μg of pAPO(a) and 5 μg of pSV-β-galactosidase control plasmids were added to incubation medium A using Calcium Phosphate Transfection Kit. After 72 h, each dish was washed twice with
medium A without FBS, and maintained in medium A with or without various stimulation. After 48 h, cell monolayers were washed twice with ice-cold phosphate buffered saline. Luciferase assay was performed by manufacture's procedure and activities were measured by luminescence using Bio-Orbid 1253 luminometer (Bio-Orbid, Turka, Finland). \( \beta \)-galactosidase activity was measured using onitrophenyl- \( \beta \)-D-galactopyranoside as substrate. Luciferase activity were normalized to \( \beta \)-galactosidase activity for each dish and standardized with that of L1 as 100%.

**Partial sequencing of 5'flanking regions of apo(a) gene**

To determine the number of TTTTA repeats and C/T polymorphism at +93 position from the transcription start site, partial DNA sequence was determined. Oligonucleotide probe A (TTTATGGTACTGTAACTGAGC) for TTTTA repeat and probe B (CTCTGAGAGAATCATTAACTTTA) for C/T polymorphism were designed according to the sequence reported by Wade et al.\textsuperscript{13}. pAPO(a) was sequenced by the Sanger dideoxy method using a Sequencing Kit and visualized by autoradiography.

**Statistical analysis**

Values were expressed as mean ± SEM. Between-group comparisons were performed by analysis of variance (ANOVA) with Bonferroni correction.

**Results**

**Lipids profile of subjects**

Table 1 shows the characteristics and the serum lipids profile of each subject. Serum Lp(a) concentrations in H1, H2 and H3 were more than 100 mg/dL and those in L1 and L2 were less than 10 mg/dL. H2 and H3 had mild hypercholesterolemia and coronary

| Table. Profile of age, presence of coronary artery disease, serum lipids, and analysis of apolipoprotein(a) in the subjects. |
|-----------------|-------|-------|-------|-------|-------|
|                 | L1    | L2    | H1    | H2    | H3    |
| Age (years old) | 30    | 33    | 25    | 57    | 60    |
| CAD             | none  | none  | none  | OMI   | AP    |
| Lp(a)(mg/dL)    | 10    | 5     | 103   | 104   | 103   |
| TC(mg/dL)       | 162   | 150   | 203   | 241   | 229   |
| TG(mg/dL)       | 116   | 214   | 131   | 119   | 159   |
| HDL-C(mg/dL)    | 49.5  | 48.7  | 33.4  | 44.0  | 21.0  |
| Apo(a)phenotype | S3/S4 | S1/S1 | S2/F  | S1/S2 | S1/S3 |
| TTTTA repeat    | 8     | 8     | 9     | 8     | 8     |
| +93 C/T         | C     | C     | C     | C     | C     |

Serum Lp(a) levels are low (<10 mg/ml) in L1 and L2, and high (>100 mg/ml) in H1, H2, H3. Abbreviations are used as follows: CAD; coronary artery disease, OMI; old myocardial infarction, AP; angina pectoris, Lp(a); lipoprotein(a) HDL-C; high density lipoprotein cholesterol, TTTTA repeat; TTTTA pentanucleotide at -1373, +93 C/T; the nucleotide at position +93 is C instead of T.
artery disease. L2 and H3 had mild hypertriglyceridemia. H1, L1 and L2 were healthy volunteers. One of the subjects with a high Lp(a) concentration, H1, had small molecular type of apo(a) protein (F-type). All had eight repeats of pentanucleotides except H1 with nine repeats. All subjects showed that the nucleotide at +93 was C. It was difficult to apply the inverse relationship between the size of apo(a) protein and serum Lp(a) levels, or between the number of pentanucleotides and serum Lp(a) levels to these results.

Promoter activity of apo(a) 5'flanking region

To investigate the activity of 5'flanking region of apo(a) gene, we constructed plasmid, pAPO(a), containing 1.4 kb fragment of this region in the upstream of luciferase gene, transfected it into HepG2 cells, and assayed their activities. Figure 1 shows the promoter activities of the apo(a) 5'flanking region without any stimulation. Promoter activities were standardized with that of L1 as 100%. L2 showed high promoter activity compared with L1, though they had low levels of serum Lp(a). H1, H2 and H3 showed subtly higher promoter activities compared with L1. However, there was no difference in promoter activities between high and low Lp(a) groups. We studied whether the polymorphism previously reported in apo(a) 5'flanking region caused this difference. The number of TTTTA repeat of H1 was nine, but the number of other subjects was eight. Every subject in our study showed C at +93 position from the transcription start. Therefore, we could not attribute the differences of basal level of promoter activity to the existence of these polymorphism.

Effect of IL-6 stimulation on apo(a) promoter activity

We investigated the effect of IL-6 stimulation on promoter activity. pAPO(a)-transfected HepG2 cells were incubated with or without 100 units/ml of IL-6 for 48 h. IL-6 stimulation induced approximately 30% enhancement of the promoter activities over the control in all subjects although not
significantly different among five subjects (Figure 2). This result suggested that these 1.4 kb fragment contain active IL-6 responsive elements.

Effects of estradiol on apo(a) promoter activity
We incubated the pAPO(a)-transfected HepG2 cells with 10 nM or 10 μM estradiol for 48 h, and apo(a) promoter activities were evaluated. The promoter activities of these 1.4 kb fragment were not altered by treatments with these agents in all cases (Figure 3).

Discussion
In this study, we prepared the 1.4 kb promoter region of the apo(a) gene from the subjects with high and low level of Lp(a), and cloned into luciferase reporter gene plasmid. Wade et al. proposed that the promoter activity of this 5’flanking regions of the apo(a) gene of relatively high plasma Lp(a) concentration was greater than that of a subject of low Lp(a) level\(^3\). However, the promoter activities of the subjects with high Lp(a) levels were not always higher than those with low Lp(a) levels in our study. The promoter activity of low Lp(a) subject L2
was rather higher than those of high Lp(a) subjects. The inverse relationship between the number of TTTTA repeat at position -1373 and serum Lp(a) concentrations is demonstrated\(^{14}\). This raises the question that the number of this pentanucleotides repeat may alter the transcription activity of apo(a) gene. In our study, H1 had nine repeats of TTTTA sequence and all other subjects had eight, but H1 did not show lower promoter activity than that of other subjects. It was demonstrated that elements essential for basal activity of apo(a) promoter were situated between -98 and +130 bp of transcription start site\(^{6,13}\). Suzuki et al. reported that three nucleotides polymorphism were existed in the 5'flanking regions of the apo(a) gene (position from -1017 to +147), and two of them were existed in the region mentioned above (C or T at +93, and A or G at +102 position from transcription start site) and induced the difference in apo(a) promoter activity\(^8\). We did not see the polymorphism at position +102, but the nucleotide at +93 was C in every case, and the promoter region which we studied included further upstream than Suzuki's report. However, no positive or negative regulation which might induce the difference of serum Lp(a) level was observed. There still exist variances among the subjects with the same apo(a) isoform and identical sequence of apo(a) allele \(^{17,19}\). Therefore, other polymorphism or sequence variation might exist in further upstream or downstream of this 1.4 kb 5'flanking region of apo(a) gene to regulate the apo(a) transcription rate in individuals.

Next, we investigated the effect of other factors, which are thought to influence serum Lp(a) level, on the promoter activity of apo(a). First, Lp(a) is reported to act as acute phase proteins in inflammation\(^{26,27}\). In the tissue of inflammation, IL-6 is considered to be excreted from peripheral blood mononuclear cells\(^{30}\). The promoter region of the apo(a) gene used here included seven IL-6 responsive elements (one is reverse consensus)\(^{13}\). In fact, IL-6 had a tendency to enhance the apo(a) promoter activity in transient transfection assay. Therefore, this region of apo(a) gene is considered to be essential to respond to the acute inflammation. Recently, Bopp et al. reported that almost identical apo(a) 5'flanking region to the sequence which we generated was not associated with the serum Lp(a) level and that longer fragments might be needed for the liver-specific Lp(a) expression\(^{31}\). We compared the enhancement of promoter activities with IL-6 among the subjects with low and high Lp(a) but there were no differences. Therefore, our data strongly support the report by Bopp et al. Second, sex hormones such as estrogen and progesterone reduce serum Lp(a) level\(^{28,29}\). In this study, we investigated the effect of estradiol on the promoter activity of the 1.4 kb apo(a) 5'flanking region, but the agent could not change the transcription rate of this region. It is likely that these agents may act synergistically with other trans-acting factor or that there may be other cis-
acting elements in further upstream or downstream of this 1.4 kb region. Recent reports described that the chromosomal region responsible for estrogen response was identified within an apo(a) enhancer located at ~26 kb from the apo(a) promoter and that binding of estrogen receptor-α to DNA was not necessary\(^3,2\).

Taken together, there may be other factors that are responsible for the regulation of the concentration of serum Lp(a) and further study will be needed to elucidate the mechanism of apo(a) transcription and protein synthesis.

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