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Expression of Apolipoprotein(a) Gene is Regulated by 5'-Haplotype rather than TTTTA Repeat

Taro Okumura, Tsukasa Osaki, Akitada Ichinose

Department of Molecular Patho-Biochemistry and Patho-Biology, Yamagata University School of Medicine

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ABSTRACT

A high plasma level of lipoprotein(a), Lp(a), is recognized as an independent risk factor for various atherothrombotic diseases. Lp(a) concentrations are under genetic control, especially by the sizes of apolipoprotein(a), apo(a). However, marked differences in plasma Lp(a) levels are present among subjects having the same apo(a) sizes. It has been reported that Lp(a) concentrations are also affected by four haplotypes (types A-D) of the apo(a) gene in its 5' -flanking region (5-FL), and a (TTTTA)n repeat (pentanucleotide repeat, PNR) upstream of these SNPs (Single nucleotide polymorphisms). Thus, we explored the relationship between 5-FL haplotypes and PNRs among Japanese individuals. Strong linkage disequilibrium was observed between the haplotypes and PNRs. 8 PNRs and 9 PNRs were associated with types A and C (8-A and 8-C) and type D (9-D), respectively. 8-C had higher Lp(a) levels than 9-D among healthy individuals and patients with ischemic heart disease. Luciferase reporter assays revealed that the difference in transcriptional efficiency of apo(a) gene between 8-C and 8-D was more distinct than that between 8-D and 9-D. Gel sift assays also suggested that 8-C had higher binding affinity to transcription factors than 9-D. Accordingly, the 5-FL haplotype rather than the PNR plays an important role in regulating plasma Lp(a) concentrations.

Key words: Apolipoprotein(a), Lipoprotein(a) level, Haplotype, Pentanucleotide repeat, Luciferase assay

Introduction

Lipoprotein(a) [Lp(a)] is a low density lipoprotein (LDL)-like particle containing apolipoprotein B-100, that is covalently attached to a high-molecular-weight glycoprotein, apolipoprotein(a) [apo(a)] by a disulfide bond[1]. Plasma levels of Lp(a) vary among normal subjects from <1 to more than 200 mg/dl over a range of 1,000-fold. This feature is not observed in other human plasma proteins. A high concentration of Lp(a) in serum/plasma is an independent risk factor for atherothrombotic diseases, such as ischemic heart disease (IHD) and cerebral infarction[2].

Apo(a) shares homologous domains with plasminogen, including multiple tandem repeats of the kringle 4 (K4) domain followed by a single copy of kringle 5[3]. Apo(a) is coded by the apo(a) gene[4] which belongs to the plasminogen-apo(a) gene family[5,6]. The apo(a) gene accounts for over 90% of the inter-individual variation in plasma Lp(a) concentrations[7]. Plasma Lp(a) levels inversely correlate with apo(a) isoform sizes; i.e. with the variable numbers of tandem K4 repeats[8,9], i.e. type 2 kringle IV (KIV-2) of the apo(a) gene. The proportion of Lp(a) variance determined by KIV-2 repeats has been estimated to be more than 40% in Caucasians[7,10].

However, even individuals having the same KIV-2 repeat show wide variation in plasma Lp(a) levels[11]. It has been reported that there could be up to a 200-fold difference in the Lp(a) concentrations associated with the same isoform[12]. Two earlier studies[13,14] also concluded that the

contribution of apo(a) size polymorphisms to the Lp(a) concentrations were only 9.7 and 9%, respectively. Plasma Lp(a) concentrations are determined not by the rate of catabolism but by its rate of production[15,16]. Thus, these results indicate that plasma Lp(a) levels may be regulated by other factors/mechanisms of the apo(a) gene, such as transcription and protein translation.

In previous studies[17,18], we reported the presence of single nucleotide polymorphisms (SNPs) in the 5'-flanking region (5-FL) of the apo(a) gene: nucleotides G/A at position -772, C/T at +93, and G/A +121, etc. We sub-classified the 5-FL of the apo(a) gene into four haplotypes (A to D) by a combination of these three SNPs: type A; G-C-G, type B; A-C-G, type C; A-C-A, type D; A-T-G. Homozygotes of type C (type CC) had significantly higher plasma Lp(a) concentrations than those of type D (type DD), and the relative expression activity of type C in an *in vitro* assay system was approximately three times higher than that of type D, which was consistent with the *in vivo* results[18]. These data suggest that the 5-FL haplotype of the apo(a) gene directly affects the transcriptional efficiency of its expression and plays a role in regulating plasma Lp(a) levels.

In addition, the number of TTTTA pentanucleotide repeats (PNRs) starting at -1373 bp (position -1231 relative to the start site of transcription) from the translation initiation codon (position +121) has been reported to have an effect on Lp(a) concentrations, which is independent of the apo(a) isoform[13,19-22]. A significant negative correlation between the number of TTTTA repeats and plasma Lp(a) levels was observed in Caucasians[19]. As to each SNP in the 5-FL of the apo(a) gene, other investigators[20] found a strong linkage disequilibrium between a SNP (i.e. +93 C/T) and the PNRs in Caucasians. However, it has been unknown which of these polymorphisms affects plasma Lp(a) levels most, and the relationship between the 5-FL haplotypes and the PNRs remains uncertain. Accordingly, the present study was carried out to elucidate the relationship between the 5-FL haplotypes and the PNRs on plasma Lp(a) levels in the Japanese population and transcriptional activity of the apo(a) gene by using a transient transfection assay and luciferase expression vectors.

Methods

Subjects and DNA samples

This study was approved by the institutional review board of the Yamagata University School of Medicine. All the procedures were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all the Japanese subjects including patients with IHD (n=85) and normal healthy controls (n=98). All IHD cases were evaluated by cardiologists. Blood samples were collected into tubes containing EDTA. Plasma was separated immediately by centrifugation, divided into aliquots and stored at -80℃ until determination of Lp(a) levels and apo(a) isoforms was carried out. Genomic DNA was prepared from leukocytes by phenol extraction.

Measurement of plasma Lp(a) levels

Plasma Lp(a) concentrations were measured by the latex-enhanced turbidimetric immunoassay method (Sanwa Chemical Laboratory, Nagoya, Japan) using an automated biochemical analyzer, COBAS MIRA Plus (Roche Diagnostics, Tokyo, Japan). One mg/dl is the lowest limit of detection according to the manufacturer's manual.

Apo(a) phenotyping

Apo(a) isoforms were determined by immunoblotting as described previously[23] with the following modifications. Five μl of plasma without delipidation was dissolved in 95μl of the sample buffer (125 mM Tris-HCl pH 6.8, containing 2% SDS, 0.02% bromophenol blue, 5% 2-mercaptoethanol, and 15% glycerol) at 100℃ for 3 min. A 10μl aliquot of this mixture was loaded onto a 8.5×8.5 cm vertical 1 mm thick gradient polyacrylamide gel with 1% SDS. A running gel composed of 3% polyacrylamide with 0.5% agarose was poured onto glass plates preheated to 60℃, and a stacking gel with 2.5% polyacrylamide was added. Electrophoresis was performed at 100 V for

3h. After electrophoresis, proteins were transferred to nitrocellulose membranes by semidry blotting for 2h at a constant current (100 mA). After protein transfer, the membrane was incubated with 5% BSA for 1h to block remaining protein-binding sites. Both a primary mouse anti-apo(a) monoclonal antibody (Terumo, Tokyo, Japan) and a secondary alkine phosphate-conjugated anti-mouse IgG antibody (E.Y Laboratory, San Mateo, CA) were diluted to 1/1000 in a blocking buffer.

Determination of the 5-FL SNPs and the number of PNRs

Each SNP (-772 G/A, +93 C/T, and +121 G/A) was determined by an amplification refractory mutation system (ARMS) and rapid automated capillary electrophoresis (RACE) method as described previously[24]. Fluorescent-labeled oligonucleotide primers for these three SNPs were designed:
5'-FAM-CAACCTCCATCTCCTGTGTACG-3' and
5'-TET-GCAACCTC CATCTCCTGTGTACA-3' for -772G/A,
5'-FAM-AAAGTGTGTCCCAA TCCCAGAACG-3' and
5'-TET-AAAGTGTGTCCCAATCCCAGAACA- 3' for +93 C/T, and
5'-FAM-ATTTTGGGACTGGCCAGCATTGC-3' and
5'-TET-ATTTTGGGACTGGCCAGCATTGT-3' for +121 G/A, respectively. The underlined bases in each primer represent the mismatch bases for ARMS. A common unlabeled antisense primer for -772 G/A was 5'-AGGCCACGGCAGATGGATCA-3', while a common sense primer for +93 C/T and +121G/A was
5'-GACTAATCAGGAAAGATG AAGGTCT-3'. To determine each genotype by ARMS, 200-500 ng of genomic DNA was amplified by PCR in a 25μl reaction mixture containing 20 pmoles of each primer and 1 unit of Taq DNA polymerase (Sawady, Tokyo, Japan). The PCR cycle consisted of the following steps: initial 3 min denaturation at 94℃, followed by 30 sec denaturation at 94℃, 30 sec of annealing at an appropriate temperature, and 30 sec of extension at 72℃ for 25 cycles. For RACE analysis, 1μl of each PCR product was mixed in a single tube with 0.5μl of GeneScan-500 TAMRA size standard (Applied Biosystems, Warrington, UK), and 15μl of deionized formamide (Amresco, Solon, OH), then heated at 95℃ for 2 min and immediately cooled on ice.

To determine the number of PNRs, a fragment including the PNRs was amplified by PCR from genomic DNA using the following primers:
5'-GAATTCA TTTGCGGAAAG-3' for sense,
5'-CGTCAGTGCACTTCAACC-3' for antisense. Amplification conditions consisted of 3 min denaturation at 94℃, followed by 30 sec of denaturation at 94℃, 30 sec of annealing at 50℃ and 30 sec of extension at 72℃ for 35 cycles. The PCR products underwent electrophoresis on 8% polyacrylamide gels at 120 V for 2.5h. Spreadex^{R} polymer NAB (Elchrom Scientific, Switzerland) was mixed into polyacrylamide gels to increase the resolving power of the gels following the manufacturer's instructions. We determined the frequency of the PNRs for each haplotype in a total of 309 Japanese subjects by combining the data of 126 individuals in the previous studies[17,18] and that of 183 individuals in the present study (Table 1).

Construction of luciferase expression plasmids

The 5-FL region of the apo(a) gene from position -1301 to +140 (relative to the start site of transcription) was amplified by PCR using subjects' genomic DNAs as templates. Oligonucleotide primers were designed to obtain two separate fragments:
5'-GAAGGTACCTGCGGAAAGATTGATACTATG-3' (TTTTA-Kpn) and
5'-TGACAGAGCAAGAATGTCTCAGGAAAG-3' (5FL-832) for amplification from -1301 to -832,
5'-CTTGAATTCCCAAAGTGCTGGGATTACAGAG-3' (A2-53) and
5'-TATGTTCCATGGTGGGACTGGCCAGCAGT-3' (5FL-Nco) for amplification from -1016 to +150 (*Kpn* I and *Nco* I sites are underlined). Each PCR fragment was inserted into an *Eco*R V-digested pBluescript vector (pBlue -1301/-832 and pBlue -1016/+150, respectively). The pBlue -1301/-832 and pBlue -1016/+150 plasmids

were digested with *Kpn* I/*Pfl*M I and *Pfl*M I/*Nco* I, respectively. These two fragments were then ligated by Ligation high^{R} (Toyobo, Osaka, Japan) and subcloned into *Kpn* I/*Nco* I-digested pGL3-Basic (Promega, Madison, WI), a promoterless luciferase reporter gene vector. Subcloned plasmids with *Kpn* I/*Nco* I-digested pGL3-B (pGL3-B -1301/+140) were designated as types A, C and D, corresponding to each of the 5-FL haplotypes[17]. The PCR products containing nucleotide positions -1016 to +150 were amplified by oligonucleotide primers, 5'-CTTGGTACCCCAAAGTGC TGGGATTACAGAG-3' (A2-53/Kpn, *Kpn* I site underlined) and 5FL-Nco, and then subcloned into *Kpn* I/*Nco* I-digested pGL3-B similarly (pGL3-B -1016/+140). The nucleotide sequences of inserts in all vectors were verified by ABI PRISM^{TM} 310 genetic analyzer (Applied Biosystems, Foster City, CA) using BigDye^{TM} terminator cycle sequencing kits (Applied Biosystems).

Cell culture and DNA transfection

HepG2 cells were maintained in culture in 60-mm dishes with low glucose Dulbecco's Modified Eagle Medium (Nikken Biomedical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (JRH biosciences, Lenexa, KS) and PSN antibiotic mixture (Gibco BRL, Gaithersburg, MD). For transient transfection, plasmids were purified by a QIAGEN plasmid maxi kit (Qiagen, Germany). 5×10^{5} HepG2 cells were co-transfected by the calcium phosphate method with 20μg of a luciferase expression plasmid and 2.5μg of a pSV β-galactosidase vector (Promega). In brief, the cells were cultured for 24h, following the addition of BES-buffered solution (pH 6.95). At 24h after transfection, the plates were washed by phosphate buffered saline (PBS) once, and replaced by the same medium. After incubation for an additional 48h, the cells were washed with PBS twice and harvested using 300μl of a lysis reagent, LCβ PGC-51 (Toyo Inki, Tokyo, Japan). Both the pGL3-B and pGL3-Control (Promega) vectors were used as negative and positive controls, respectively, for the expression experiments.

Assays of luciferase and β-galactosidase activity.

For measurement of luciferase activity, a luminometer, Lumat LB 9507 (EG&G Berthold, Germany), was employed by mixing 20μl of the cell lysates and 100μl of the luciferase substrate solution (Toyo Inki). To determine β-galactosidase activity, 20μl of cell lysates were mixed in a 96-well plate with 220μl of 0.05M potassium phosphate buffer (pH 7.8), 30μl of 0.01M $MgCl_2$, 40μl of 10 mM *o*-nitrophenyl-β-D-galactopyranoside (Sigma), and 3μl of 8.9M 2-mercaptoethanol. The plate was incubated at room temperature for 30 min and measured by absorption at 405 nm using Biolumin 960 (Molecular Dynamics, Sunnyvale, CA). Luciferase activity was normalized to β-galactosidase activity for each dish.

Preparation of nuclear extracts

Nuclear extracts were prepared from 1×10^{7} HepG2 cells. After washing with PBS twice, the cells were lysed by 0.4 ml of a cytoplasmic lysis buffer (20 mM HEPES pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, and 20% glycerol), and centrifuged, then a supernatant fraction was removed. A nuclear pellet was incubated on ice for 30 min after adding 0.1 ml of nuclear lysis buffer (20 mM HEPES pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, and 20% glycerol) and centrifuged. The supernatant was collected as nuclear extracts, aliquoted into tubes, and stored at -80℃. Protein concentrations were measured by a BCA protein assay reagent (Pierce, Rockford, IL) following the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Radioactively labeled DNA probes were prepared by PCR. The luciferase expression vector containing the romoter region of the apo(a) gene was amplified using the primers as follows: TTTTA-Kpn and 5FL-Nco (^{32}P -1301/+150), where an appropriate amount of [α-^{32}P] dCTP (NEN Life Science Products, Boston, MA) was added to the PCR mixture. Then, the PCR products were purified using Quantum prep PCR kleen spin columns (Bio-Rad, Hercules, CA). Binding reaction was performed using 2 μg of

poly dI-dC, 2 μg of nuclear extracts, 1 x 10^4 cpm ^{32}P labeled DNA probe, and a reaction buffer [25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA (pH 8.0), 50 mM KCl, 10% glycerol, 0.5 mM DTT and 0.5 mM PMSF]. The reaction mixture was incubated for 30 min at room temperature. After preheating for 1 h at 100 V, EMSA was performed on a 4% non-denaturing polyacrylamide gel in 0.5 x TBE for 2 h at 4 ℃. Gels were subjected to autoradiography by a fluoro-image analyzer FLA-2000 (Fuji Film, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed with a Stat View program (SAS Institute, San Francisco, CA) and SAS enterprise guide 4.3. Linkage disequilibrium was determined by a chi-square test. Linear regression analyses were performed to assess the relationship between Lp(a) levels and apo(a) phenotypes. All values concerning Lp(a) levels are shown in means±standard deviation (S.D.). Lp(a) concentrations between different groups were examined by the Kruskal-Wallis test. For a luciferase assay and EMSA, a paired t-test was performed. A p-value of <0.05 was considered to be statistically significant.

Results

Relationship between plasma Lp(a) levels and the 5-FL haplotypes

Lp(a) levels were 13.5±12.3 and 19.3±18.4 mg/dl (p=0.034) in healthy individuals (Normal, n=98) and cases with IHD (n=60), respectively. When two groups were combined, individuals of type CC (n=43) had significantly higher Lp(a) levels than those with type DD (n=9); 18.9±20.9 vs.10.5±6.9 mg/dl (p=0.038), as reported earlier[18]. We also compared plasma Lp(a) levels of each haplotype in two groups. Lp(a) levels of type CC in IHD (n=22) were two-fold higher than those of type DD (n=6, 26.3±26.0 vs.13.2±6.7 mg/dl, respectively), and the difference was statistically significant (p=0.04). This tendency was also shown in Normal (11.2±9.1 vs. 5.1±3.3 mg/dl, p=0.28), although statistical significance was not obtained. It was of note that six subjects having markedly high plasma Lp(a) levels (greater than 50 mg/dl) were present in the IHD group, and five of them were homozygous for type C.

Relationship between Lp(a) levels and apo(a) sizes

As reported previously[8,9], Lp(a) concentrations were found to correlate inversely with apo(a) protein sizes in Normal and IHD groups in the present study (data not shown). Especially, the lower-molecular-weight (L-MW) isoform among two apo(a) isoforms of each individual was more closely correlated with plasma Lp(a) levels than the higher-molecular-weight isoform, as reported previously[23]. Therefore, we examined the correlation between Lp(a) concentrations and L-MW isoforms in all groups and IHD patients having type CC and DD. We did not draw a figure for Normal because a very limited number of subjects with type DD (n=3) were available. Fig. 1A & C showed that plasma

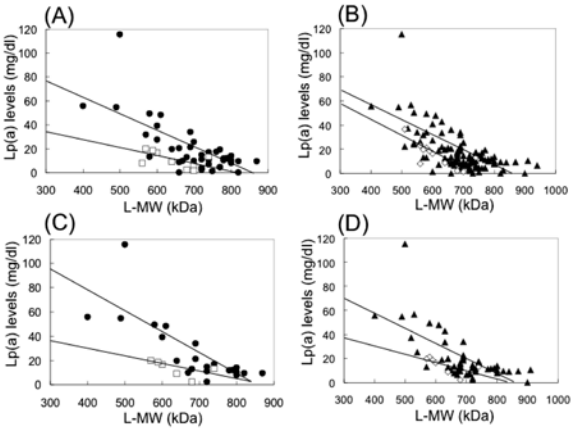

Fig. 1. Relationship between plasma Lp(a) levels and lower-molecular-weight (L-MW) apo(a) isoforms of individuals with haplotype CC (closed circles) or DD (open squires) in total (**A**) and in patients with IHD (**C**). For IHD, the regression line for type CC (n= 22): Lp(a) levels = 143.44 – 0.17 × L-MW, R^2= 0.61, p<0.0001. The regression line for type DD (n= 6). Lp(a) levels = 54.42 – 0.06 × L-MW, R^2= 0.38, p= 0.19. **Relationship between plasma Lp(a) levels and apo(a) L-MW isoforms in cases with 8/8 (closed triangles) and 9/9 ≦ (open diamonds) for the PNRs** in total (**B**) and in patients with IHD (**D**). For IHD, the regression lines indicate for 8/8 and 9/9 ≦ for the PNRs, respectively. For 8/8 (n= 53) : Lp(a) levels = 109.41 – 0.13 × L-MW, R^2= 0.46, p<0.0001, for 9/9 ≦ (n= 7): Lp(a) levels = 60.89 – 0.074 × L-MW, R^2= 0.45, p= 0.10. An inverse relationship was demonstrated between Lp(a) levels and L-MW isoforms both for the 5-FL haplotypes and PNRs.

Lp(a) levels were inversely correlated with apo(a) isoforms in both haplotypes. Lp(a) levels of type CC were higher than those of type DD, even when they had similar apo(a) sizes. These results are in concordance with the conclusion that apo(a) expression is affected by haplotypes, as previously reported[18].

We then examined the relationship between Lp(a) levels and apo(a) sizes in homozygotes of 8 PNRs (8/8) with those of equal to or greater than 9 PNRs (9/9≦) in all individuals and in IHD patients. The 9/9≦ PNRs contained homozygotes for 9 (9/9), heterozygotes for 9 and 10 (9/10), and heterozygotes for 9 and 11 (9/11). Lp(a) concentrations of the 8/8 PNRs were higher than those of the 9/9≦ genotypes in the subjects having similar apo(a) isoforms (Fig. 1B & D). However, the difference caused by the PNRs was less distinguished than the difference caused by the haplotypes.

In order to examine the effect of PNRs on plasma Lp(a) levels in Japanese, we directly compared Lp(a) levels of three different PNR groups (8/8, 8/9 and 9/9≦). Lp(a) concentrations did not differ significantly with respect to the PNRs in total, Normal and patients with IHD (Fig. 2A-C; p=0.93-0.08).

Table 1. Numbers of the TTTTA-PNR among each haplotype in Japanese.

Frequencies are shown in parentheses. Linkage disequilibrium was observed between the PNRs and the 5-FL haplotypes. * ; p= 0.0004, ** ; p < 0.0001.

PNR \ Haplotype	AA	CC	DD	AC	AD	CD	AB/BC	Total
8/8	32 (1.00)**	67 (0.97)**		77 (0.94)**		1 (0.02)		177
8/9			1 (0.06)	5 (0.05)	39 (0.75)**	48 (0.90)**		93
8/10		2 (0.03)			3 (0.06)	3 (0.06)	3 (1.00)	9
8/11				1 (0.01)				3
9/9			12 (0.70)*		10 (0.19)			22
9/10			4 (0.24)					4
9/11						1 (0.02)		1
Total	32	69	17	83	52	53	3	309

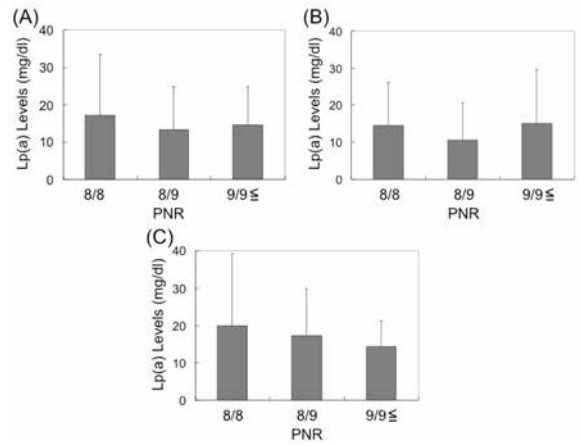

Fig. 2. Plasma Lp(a) levels in a total of 178 individuals (A), 93 normal individuals (B), and 85 patients with IHD (C) as a function of the number of PNRs. There was no statistical difference in Lp(a) levels between samples with different numbers of PNRs.

Association between the 5-FL haplotypes and the PNRs

Since Lp(a) concentrations were related to both the 5-FL haplotypes and the number of PNRs, we determined the frequency of the PNRs for each haplotype in 309 Japanese subjects combining the data of previous studies[17,18] (Table 1). Most subjects having type CC and homozygotes of type A contained the 8/8 genotype for PNRs; on the other hand, 70% of individuals with the type DD possessed 9/9 genotype. Therefore, an allele with 8 PNRs was associated with types A and C, whereas a 9 PNRs allele linked to type D. Thus, there is a statistically significant association between each

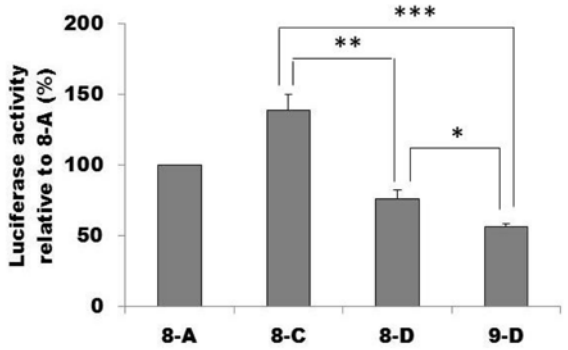

Fig. 3. Effects of PNRs and haplotypes on transcriptional activity of the apo(a) promoter region. HepG2 cells were co-transfected with test plasmids and a β-galactosidase vector as a control of transfection efficiency. Luciferase activity was indicated relative to that of haplotype A with PNR-8 (8-A; 100%). Values represent means±S.D. of 3 independent experiments. A p-value of <0.05 was considered to be statistically significant. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$.

haplotype and a certain number of PNRs (Table 1). An allele with 10 PNRs seemed to be related to type B, but the number of subjects was too small to draw a conclusion (only 3 heterozygotes of type B among 309 subjects). All cases having an allele with 11 PNRs were heterozygotes for type C. Accordingly, strong linkage disequilibrium between the 5-FL haplotypes and the PNRs was observed in this study.

Effect of the haplotypes and PNRs on transcriptional activity

To elucidate the relationship between the 5-FL haplotypes and the PNRs *in vitro*, we studied the promoter activity of 8-A, 8-C, 8-D and 9-D by a transient transfection assay. 8-D had 1.4-fold higher luciferase activity than 9-D (p=0.01) (Fig. 3). However, 8-C had 1.8-fold higher than 8-D (p=0.003) and 2.5-fold than 9-D (p=0.0006). This data indicates that 5-FL haplotypes affect the transcriptional efficiency of the apo(a) gene.

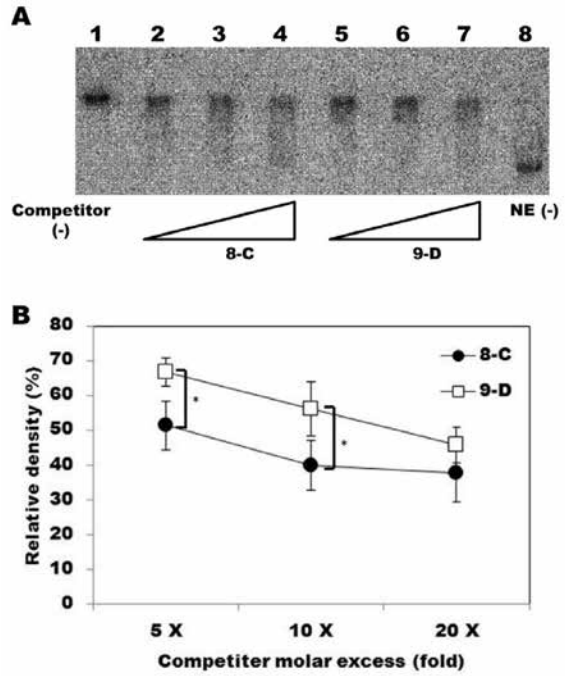

Fig. 4. Inhibition by competitor supplementation on labeled type A probe. (A) EMSA for 5-FL of the apo(a) gene (-1301/+150). Labeled PCR products fortype A having the eight TTTTA repeats were incubated with 2 μg of nuclear extracts prepared from HepG2 cells (lane 1). In lane 8, no nuclear extracts were contained in a reaction mixture (negative control). For competition analysis, an unlabeled PCR fragment was added to labeled type A probe in 5, 10 and 20-fold molar excess (lane 2 and 5, 3 and 6, and 4 and 7, respectively). Number of TTTTA repeat used as unlabeled type C and D fragments were eight (8-C) and nine (9-D), respectively.
(**B**) Amounts of bound probe are expressed as a percentage of relative density to labeled type A probe (lane 1 in Fig 4a). Values represent means±S.D. of 5 independent experiments. Quantitative analysis was performed by a fluoro-image analyzer FLA-2000. Asterisks illustrate statistically significant differences between type C and type D (*, $p<0.05$).

Differential DNA-protein binding among the haplotypes

From the results shown above, we hypothesized that varying binding affinity to transcription factors would cause the difference of luciferase activity among the haplotypes. To test this hypothesis, we carried out an EMSA to examine which haplotype of type C and D is more competitive to labeled type A probe. Non-labeled DNA fragment of type C more significantly reduced the binding ability on labeled probe (^{32}P -1301/+150) as compared to those of type D (Figs. 4A & B). The ability of type A to compete for binding of nuclear proteins showed a halfway between type C and D (data not shown).

Discussion

Apo(a) gene expression is regulated mainly by liver-enriched Hepatocyte Nuclear Factor 1α (HNF-1α)[25]. It is also reported that the 1.4 kb apo(a) 5-FL comprises two composite regulatory regions: a distal negative regulatory module (positions -1432 to -716) and a proximal tissue-specific module (positions -716 to -616)[26]. The former module contains the polymorphic PNR at position -1231 and the latter locates consensus sequences for hepatocyte-specific transcription elements such as LF-A1, CEBP, and HNF-1α[6], which is consistent with the fact that the apo(a) gene is exclusively expressed in the liver. These cis-elements in the 5-FL and KIV-2 repeat control the Lp(a) levels in plasma[27].

As reported earlier, several SNPs (-772 G/A, +93 C/T, and +121 G/A) present in the 5-FL of the apo(a) gene comprise four haplotypes (A-D) [17], while the PNR exists upstream from these SNPs.

We demonstrated that the 5-FL haplotypes in part regulate apo(a) gene expression[18]. However, plasmid constructs in our previous experiments did not contain the region of the PNR.

A number of studies demonstrated that the number of PNRs was negatively correlated with Lp(a) levels *in vivo*[13,19-22,28,29]. However, no difference was found in the transcriptional activity of 5-FL fragments containing 8 or 11 PNRs of the apo(a) gene *in vitro* where haplotypes of the fragments were not identified[30]. This is consistent with the report that 10 allelic apo(a) 5-FL fragments of 1.5 kb containing 8 or 9 PNRs exhibit comparable promoter activities in HepG2 cells, although only the SNP at position -772 was specified[31]. In the present study, we have clearly shown that the 5-FL haplotype rather than the PNR plays an important role in regulating the transcription of apo(a) gene (Fig. 3). Similar PNRs are also present in other human genes, for example, the β-globin gene[32], where there are 4 to 6 repeats about 1.4 kb upstream from the ATG initiation codon as in the apo(a) gene. This variation in the number of PNRs has no effect on β-globin promoter activity.

The reason why the number of PNRs is negatively correlated with Lp(a) levels in the previous reports, including European Caucasians, Japanese and Chinese, needs to be addressed. It is likely that PNRs are in strong linkage disequilibrium with the 5-FL haplotypes of apo(a) that affect its transcriptional activity; for example, 9 PNR is associated with the +93T SNP (Table 1), which is identical to Japanese haplotype D with the lowest transcriptional activity *in vitro*[17,18]. The strong linkage disequilibrium between 9 PNR and the T allele has also been reported in white Caucasians and Asian-Indian[33], European Caucasians[20], and Korean[34]. This +93C/T polymorphism, which introduces an upstream ATG codon and reduces *in vitro* translation[35], showed a significant impact on Lp(a) levels in black Africans but not in Caucasians[36]. Alternatively, it is reported that there is significant linkage disequilibrium between the number of PNRs and that of KIV-2 repeats[30,36] which are associated with particular Lp(a) levels. Experimental data have shown that the processing and secretion of apo(a) is a function of the number of KIV-2 repeats *in vitro*[37]. However, this assumption on the translational regulation is not in agreement with previous reports that the effect of the 5-FL PNRs on plasma Lp(a) concentrations was independent from the KIV-2 repeat/apo(a) size polymorphism *in vivo*[19,20,22]. Moreover, it has been shown that the PNR elements containing 5, 8, and 10 repeats bind differently to transcription factors in a hepatocyte-specific manner[26], indicating that the effect of PNRs on plasma Lp(a) levels is mediated at least in part by apo(a) gene transcription rather than by its translation.

It is of note that the 5-FL of the chimpanzee apo(a) is 98% homologous to its human counterpart, and contains only 4 stable repeats for PNRs in common in the 1.4 kb region[38]. The chimpanzee promoter with 4 PNRs exhibited 5-fold transcriptional activity to its human counterpart, while the mean plasma Lp(a) levels in the chimpanzee are more than 3 times higher than those observed in humans[39].

The nucleotide sequence of the apo(a) gene varies not only inter-individually but also between racial groups, e.g. SNPs/haplotypes[17,33] and PNR[19] in the 5-FL, KIV-2 repeat[40,41] and coding sequences in exons[42], and so on. Accordingly, it is essential to examine KIV-2 repeats/SNPs in each racial group in order to understand the relationship between genomic nucleotide sequences and their effects on the expression of the apo(a) gene under physiological and/or pathological conditions.

Little is known about population-related SNPs in the apo(a) gene among Japanese. Although the difference of plasma Lp(a) levels between IHD patients with type CC and those with DD was not statistically significant, most subjects having markedly high plasma Lp(a) levels were homozygous for type C. These results suggest that the frequency of type CC may be high among subjects having markedly elevated Lp(a) levels. As expected, significantly elevated Lp(a) levels were also observed in Japanese patients with corticosteroid-induced osteonecrosis of the femoral

head who had type CC[43].

Several studies suggested the 5-FL region of the apo(a) gene may mediate response to drugs and hormones. For example, retinoids were reported to lower apo(a) mRNA levels in primary hepatocyte cultures and a retinoid response element at -1036 was shown to be responsible for this effect[44]. Aspirin also reduced apo(a) levels in a culture medium of human hepatocytes, and a promoter region extending from -30 to +138 was critical for this effect of aspirin[45]. Accordingly, mutations and SNPs in the 5-FL of the apo(a) gene may lead to differential responses to drugs and physiological substances among individuals, an important consideration for medicines that are designed specifically to target the advance of atherosclerosis and development of thrombosis. Since mutations/SNPs are fairy different between racial groups[17,36], it is important to examine them in each population.

There are several limitations in our present study. First, we have investigated only Japanese subjects, no Caucasians or black Africans. Mean Lp(a) levels in black African are known to be significantly higher than in other populations, and the apo(a) alleles with lower number of PNRs are more frequent in black Africans[19]. Accordingly, the relationship between 5-FL haplotypes and PNRs could be different in other racial groups, as well. Second, other research groups[46,47] demonstrated that two additional apo(a) regulatory sites existed further upstream from the region we investigated in the present study. There is a possibility that these remote sequences may interact with the 5-FL haplotypes or PNRs, and affect apo(a) gene expression. That being the case, the relationship between SNPs in these upstream sites to the transcriptional activity of the apo(a) gene has been demonstrated *in vitro*[48]. In particular, alleles with a G variant were associated with 70% higher Lp(a) levels than those with an A variant, and these A/G SNPs were in linkage disequilibrium with the -772G/A and +93C/T SNPs in the apo(a) promoter. Nevertheless, all the known polymorphisms are not enough to explain the differences of over 200-fold in Lp(a) concentrations associated with apo(a) alleles of the same size. Recently, it has been reported that eight SNPs that were not associated with KIV-2 repeats were associated with Lp(a) concentration, suggesting that the SNPs are related to an alternative mechanism for modifying Lp(a) concentration, such as efficiency of transcription or expression[49]. In addition, a meta-analysis has recently revealed no candidate genes outside the apo(a) gene to have an effect on Lp(a) levels[50].

In conclusion, we have demonstrated that the 5-FL haplotypes rather than the PNRs in the apo(a) gene's promoter region directly control its expression. We have also shown that PNRs are merely related by linkage disequilibrium with the 5-FL haplotypes. The haplotypes may have some clinical significance, if differential responses to drugs and inflammatory agents were observed in patients. Further investigations will be carried out in order to explore the possible effects on apo(a) gene expression of Lp(a)-lowering drugs, cytokines and hormones among the hapolotypes.

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