Low Molecular Weight Phenotype of Apolipoprotein(a) Is a Risk Factor of Corticosteroid-Induced Osteonecrosis of the Femoral Head After Renal Transplant

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ABSTRACT. ***Objective.*** Osteonecrosis of the femoral head (ONF) is a necrosis due to disruption of the blood flow. The disease often occurs in association with corticosteroid treatment. The pathology of corticosteroid-induced ONF is unclear, although abnormalities in the coagulation and fibrinolytic systems or in the lipid metabolism have been reported to be involved. We examined the relationships between development of ONF and genetic variations and plasma level of lipoprotein(a) (Lp(a)), which is closely involved in the coagulation and fibrinolytic systems and lipid metabolism.

Methods. The study population consisted of 112 renal transplant patients undergoing corticosteroid treatment. Their apolipoprotein (a) [apo(a)] isoform was determined by Western blotting, and patients were classified into low molecular weight (LMW) or high molecular weight (HMW) groups. The plasma Lp(a) level was measured. Patients were also examined for 3 single-nucleotide polymorphisms (SNP), –773 (G/A), +93 (C/T), and +121 (G/A). Relationships between these 3 genetic factors of Lp(a) and ONF development were examined using statistical methods including multivariate analysis.

Results. A strong relationship was observed between the apo(a) molecular weight phenotype and ONF development, with an increased risk of ONF development for the LMW group (adjusted odds ratio 5.75, 95% CI 1.76–18.74, p = 0.0038). No significant relationships were observed between ONF and plasma Lp(a) level and SNP.

Conclusion. Apo(a) molecular weight phenotype would be a useful predictor of ONF that develops after corticosteroid treatment. (First Release Dec 1 2006; J Rheumatol 2007;34:516–22)

Key Indexing Terms:
CORTICOSTEROIDS OSTEONECROSIS LIPOPROTEIN(a)
PHENOTYPE POLYMORPHISM

Nontraumatic osteonecrosis of the femoral head (ONF) is an intractable disease that is pathophysiologically characterized by ischemic necrosis of femoral head and deterioration of hip joint functions, and these changes significantly affect quality of life of the patient[1]. Clinical application of corticosteroids and alcoholism are known as causative factors, and in particular, corticosteroid is reported to induce ONF at a high frequency[1]. Pathophysiological features of corticosteroid-induced ONF are as follows: there is development very early in the treatment period[2]; the necrotic lesion can be significantly large, which makes preservation of the femoral head difficult and requires quite invasive total hip replacement; and it is an iatrogenic disease. Therefore, it is important (1) to identify high-risk patients before starting corticosteroid treatment of the basic disease; (2) to tailor individual corticosteroid dosage in order to reduce the incidence of ONF; and (3)

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to detect occurrence of corticosteroid-induced ONF at an early stage in every patient through frequent screening. The pathology of corticosteroid-induced ONF has not been fully clarified, but it is thought that drastic ischemic changes occur in the femoral head and then bone necrosis develops[1].

As a cause of ischemic changes in the femoral head, a relationship between abnormal lipid metabolism and corticosteroid-induced ONF has been reported[3-5]. The relationship of the lipid molecule lipoprotein(a) (Lp(a)) with various vascular lesions has attracted the attention of researchers in the thrombotic and ischemic diseases. Several studies have reported a relationship between plasma Lp(a) concentration and vascular lesions such as coronary heart disease, stroke, and carotid atherosclerosis[6-9], and the level of Lp(a) has also been related to Perthes' disease[10,11] and bone marrow edema[12].

Lp(a) is a low-density lipoprotein (LDL)-like lipoprotein that has a component of 2 disulfide-linked high molecular weight proteins, apolipoprotein(a) (apo(a)) and apolipoprotein B-100. Apo(a) has a reiterated sequence of kringle 4, it is structurally quite similar to human plasminogen, and it is considered a lipoprotein that induces both arteriosclerosis and thrombogenesis[13]. Glueck, *et al* reported high serum Lp(a) levels in Caucasian patients with ONF[14]. On the other hand, recent studies report that Lp(a)-induced thrombogenesis and progression of arterial sclerosis are influenced by the molecular weight of Lp(a) that is determined by the kringle 4 repeat number and not by its level in serum[15,16]. The serum level is determined secondarily to the differences in translation efficiency in the liver, and this difference is induced by the molecular weight[17-19]. This suggests that individual differences in the molecular weight as well as the serum concentration of apo(a) would influence ONF development.

Molecular weight phenotype is determined by size polymorphism in the gene encoding the apo(a) protein, and it is genetically confined, stable, and not affected by environmental factors[20]. It is hypothesized that molecular weight phenotype relates to ONF development, while plasma Lp(a) level has no influence on the occurrence of ONF. On the other hand, plasma Lp(a) levels in Japanese people are reported to be affected by the haplotype that is the integration of 3 gene polymorphisms in the promotor region[21], although this haplotype is thought to have no influence on molecular weight phenotype.

We examined the relationship between apo(a) isoforms with different molecular weights or plasma Lp(a) levels and corticosteroid-induced ONF after renal transplants in Japanese patients. We also examined whether haplotype was related to the development of corticosteroid-induced ONF.

MATERIALS AND METHODS

Study design. Osteonecrosis after renal transplant is one expression of corticosteroid-induced osteonecrosis[22]. We examined 112 patients who received renal transplants in our university hospital between 1983 and 2004. The total number of transplant patients in this period was 428; 316 patients who had any of the following conditions were excluded: those whose transplanted kidney lost normal function and in whom dialysis was started; those who had been diagnosed as having ONF before renal transplant; those whose magnetic resonance imaging findings did not satisfy the diagnosis criteria (e.g., band-like low signals in the femoral head in T1-weighted images); those who had hip joint disease such as acetabular dysplasia or osteoarthritis before renal transplant; those who received renal transplants before the introduction of cyclosporine in 1982; and those who declined to participate in the study.

Among 112 patients examined, 20 developed ONF (ONF group) and 92 did not develop ONF (the reference group) during their postoperative monitoring period. ONF was diagnosed according to published criteria[23]. Reference patients had received transplants more than 1 year before the start of the study and were confirmed to have no osteonecrosis in the hip, knee, shoulder, or ankle joint.

The study was approved by the ethical review board on human genome/gene analysis research of our university, and written informed consent was obtained from each participating patient.

Clinical information. All patients received intravenous (IV) injection of methylprednisolone 500 mg during the surgery and IV injection of prednisolone (PSL) 50 mg on the day of surgery. Starting the following day, the patient received oral administration of PSL 50 mg/day for 7 days or 3 days, then 40 mg/day for 7 days or 4 days. The dose was reduced to 30, 25, 20, and 17.5 mg/day every 7 days, and then to 10 mg/day 6 months later. The total oral dosage of each patient up to the fourth postoperative week was 924.6 mg on average.

Analysis of apo(a) isoforms and molecular weight phenotypes. Plasma samples were collected from the 112 patients for Western blotting. Following the method of Utermann, *et al*[24], the apo(a) molecule was classified into one of the 6 isoforms, i.e., F, B, S1, S2, S3, and S4, by Western blotting that utilized sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight is approximately 400 kDa for isoform F, 460 kDa for B, 520 kDa for S1, 580 kDa for S2, 640 kDa for S3, and 700 kDa for S4[19]; the weight is determined by the kringle 4 repeat number. Based on the conventional classification, Wahn, *et al*[25] determined F, B, S1, and S2 as low molecular weight (LMW) isoforms, and S3 and S4 as high molecular weight (HMW) isoforms, and compared longterm graft survival in organ recipients retrospectively. Utermann, *et al*[24] investigated the relationship between plasma concentrations and molecular weights, finding a cutoff level between S2 and S3, and classified F, B, S1, and S2 as LMW, and S3 and S4 as HMW.

In our study, the patients were classified into 3 groups, i.e., MW phenotype LL that possesses the L isoform only (e.g., S1S1, FS2); MW phenotype LH that possesses L and H isoforms (e.g., BS3, S1S4); and MW phenotype HH that possesses the H isoform only (e.g., S3S3, S3S4). At the same time, patients were also classified into 2 other groups[26] — the LMW group that possessed at least one LMW isoform, or the HMW group that possessed only the HMW isoform. The frequency of ONF in relation to apo(a) molecular weight phenotype was examined statistically.

Measurement of plasma Lp(a) levels. Plasma Lp(a) levels were measured by latex agglutination immunoassay[27]. Peripheral blood was sampled in the period 13 to 268 months after renal transplant (mean 106 mo, steady-state) in the morning before breakfast. The frequency of ONF in relation to plasma Lp(a) level was examined statistically.

Sequence analysis of SNP in the apo(a) gene and gene haplotype classification. Gene analysis was conducted on 105 of the 112 patients in order to examine G(–773)A, C(+93)T, and G(+121)A, the SNP in the 5' flanking region of the apo(a) gene that are reported to relate to plasma Lp(a) levels[28,29]. Genomic DNA was obtained from peripheral blood using the DNeasy™ tissue kit (Qiagen GmbH, Hilden, Germany).

Polymerase chain reactions (PCR) and sequencing of purified PCR fragments were performed according to the method described by Asano, *et al*[30].

In addition, according to the combinations of the 3 SNP, haplotypes were classified into 4 types: A [G(–773), C(+93), G(–21)]; B [A(–773), C(+93), G(–21)]; C [A(–773), C(+93), A(–21)]; and D [A(–773), T(+93), G(–21)][21,28], and their relationship to development of ONF was examined.

MW phenotype and plasma Lp(a) level, and plasma Lp(a) level and gene hap-

lotype. Relationships between MW phenotype and plasma Lp(a) level were examined in the 112 patients, and relationships between plasma Lp(a) level and gene haplotype were examined in the 105 patient subgroup.

Statistical methods. Statistical significance was assessed by chi-square test, Fisher's exact test, Wilcoxon's rank-sum test, Student t test, or the Mantel extension method. The crude and adjusted odds ratios (OR) and their 95% confidence intervals (CI) were calculated using the logistic regression model. Test for trend was performed by applying exposure variables coded by ordinal numbers with increasing dose levels into the model. Analyses were all conducted using the Statistical Analysis System (SAS, v. 9.0). P values < 0.05 were considered statistically significant.

RESULTS

Relationship between patients' general characteristics and ONF. Development of ONF was not related to patient's sex, age at surgery, immunosuppressant therapy after renal transplant, type of transplanted kidney, presence or absence of acute rejection, and corticosteroid dose. In the logistic regression model, univariate analysis showed no relationship between each characteristic and ONF development, while multivariate analysis showed a significant relation between age and ONF development (adjusted OR 1.06, 95% CI 1.00–1.11, p = 0.035).

Relationship between apo(a) MW phenotype and ONF (Table 1). ONF occurred in 2 of the 5 LL patients (40.0%), 8 of 19 LH patients (42.1%), and 10 of 88 HH patients (11.4%). A strongly significant difference (p = 0.0016) was observed among LL, LH, and HH patients with the Mantel extension method. In the LMW group that combined LL and LH, ONF occurred in 10 of the 24 patients (41.7%), while ONF occurred in 10 of 88 HMW patients (11.4%), a strongly significant difference (p = 0.0016). These data showed that risk of ONF was significantly high in the patients having a small MW isoform. The LH group had significantly higher risk of ONF than the HH group in both univariate and multivariate logistic regression analyses that included sex, age, immunosuppressant, type of transplanted kidney, presence or absence of acute rejection, and corticosteroid dose (crude OR 5.67, 95% CI 1.84–17.45, p = 0.0025; adjusted OR 5.98, 95% CI 1.57–22.74, p = 0.0087). The average of total oral doses up to the first 4 weeks was 923.3 mg in the LMW group, and 925 mg in the HMW group. There was no significant difference between these doses (p > 0.05, Student t test). In the LMW group, the OR increased significantly in comparison to the HMW group in univariate and multivariate analyses (crude OR 5.57, 95% CI 1.96–15.84, p = 0.0013; adjusted OR 5.75, 95% CI 1.76–18.74, p = 0.0038). These data are important because they show that the risk of development of ONF increases when one of the LMW isoforms, i.e., F, B, S1, and S2, is included in the phenotype.

Relationship between plasma Lp(a) level and ONF. The mean, median, and range of plasma Lp(a) levels were 19.4 mg/dl, 14.0 mg/dl, and 1–66 mg/dl in the ONF group, while they were 12.9, 6.0, and 1–85 in the reference group. Levels were higher in the ONF group, but there was no significant difference (p = 0.056, Wilcoxon rank-sum test).

Relationship between apo(a) gene haplotype and ONF (Table 2). The haplotype frequencies were 31.3% for group A, 0.7% for B, 41.3% for C, and 26.7% for D. These were almost the same as the frequencies in a Japanese population reported in a previous study[28]. Table 2 summarizes the results of univariate and multivariate analyses on the relation between haplotype and ONF. No statistically significant relationship was observed.

Relationship between apo(a) MW phenotype and plasma Lp(a) level. A significant difference was found in the mean plasma Lp(a) levels: 33.8 mg/dl in the LMW group and 8.7 mg/dl in the HMW group (p < 0.0001, Wilcoxon rank-sum test). This agrees with the data in previous reports[21,26], and shows the molecular weight of apo(a) is inversely associated with the plasma Lp(a) level.

Relationship between plasma Lp(a) level and apo(a) gene haplotype (Figure 1). In order to confirm that apo(a) gene haplotype is the gene polymorphism that affects plasma Lp(a)

Table 1. Apolipoprotein (a) molecular weight (MW) phenotype and the risk of ONF. Values for ONF and reference group are number (%).

	Group			Odds Ratio			
	ONF, n = 20	Reference, n = 92	p	Crude OR (95% CI)	p	Adjusted OR* (95% CI)	p
Molecular weight phenotype							
LL	2 (40.0)	3 (60.0)	0.0016†	5.20 (0.77–34.99)	0.0901	5.17 (0.69–38.72)	0.1097
LH	8 (42.1)	11 (57.9)		5.67 (1.84–17.45)	0.0025	5.98 (1.57–22.74)	0.0087
HH	10 (11.4)	78 (88.6)		1 (trend: p = 0.00389)		1 (trend: p = 0.0076)	
LMW	10 (41.7)	14 (58.3)	0.0016‡	5.57 (1.96–15.84)	0.0013	5.75 (1.76–18.74)	0.0038
HMW	10 (11.4)	78 (88.6)		1		1	

Statistical analyses performed using † the Mantel extension method, ‡ Fisher's exact test. L: Low MW isoform (F, B, S1, S2). H: High MW isoform (S3, S4). LL: combinations of L alone (e.g. S1S1, FS2). LH: Combinations of L and H (e.g. BS3, S1S4). HH: combinations of H alone (e.g. S3S3, S3S4). LMW (low MW): containing at least 1 L isoform. HMW (High MW): without any L isoform. * This model includes gender, age, immunosuppressant, kidney, acute rejection, corticosteroid dose as well as MW phenotype.

Table 2. Haplotype classification and the risk of ONF. Haplotypes are: A: G (–773), C (+93), G (+121); B: A (–773), C (+93), G (+121); C: A (–773), C (+93), A (+121); D: A (–773), T (+93), G (+121).[28]

	Group		Odds Ratio			
	ONF, (n = 18)	Reference, (n = 87)	Crude OR (95% CI)	p value	Adjusted OR* (95% CI)	p value
Haplotype						
A+A	1 (10.0%)	9 (90%)	0.83 (0.09–7.79)	0.873	0.76 (0.07–8.09)	0.821
A+B or C or D	11 (25.0%)	33 (75.0%)	2.50 (0.84–7.45)	0.099	1.85 (0.55–6.22)	0.321
B/C/D+B/C/D	6 (11.8%)	45 (88.2%)	1 (trend: p = 0.424)		1 (trend: p = 0.706)	
B+B	0	0	—	—	—	—
B+A or C or D	1 (50.0%)	1 (50.0%)	5.06 (0.30–84.89)	0.260	0.908 (0.04–22.19)	0.953
A/C/D+A/C/D	17 (16.5%)	86 (83.5%)	1		1	
C+C	3 (12.5%)	21 (87.5%)	0.43 (0.10–1.78)	0.244	0.66 (0.13–3.31)	0.614
C+A or B or D	6 (13.3%)	39 (86.7%)	0.46 (0.15–1.45)	0.185	0.74 (0.19–2.85)	0.657
A/B/D+A/B/D	9 (25.0%)	27 (75.0%)	1 (trend: p = 0.177)		1 (trend: p = 0.587)	
D+D	2 (20.0%)	8 (80.0%)	1.35 (0.25–7.32)	0.728	1.13 (0.15–8.47)	0.903
D+A or B or C	6 (19.4%)	25 (80.6%)	1.30 (0.42–3.96)	0.649	1.25 (0.33–4.70)	0.743
A/B/C+A/B/C	10 (15.6%)	54 (84.4%)	1 (trend: p = 0.624)		1 (trend: p = 0.800)	

* Adjusted for gender, age, immunosuppressant, kidney, acute rejection, corticosteroid dose and molecular weight phenotype.

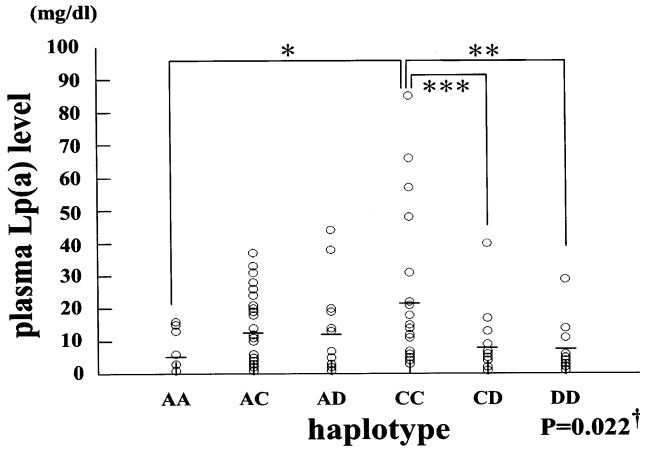

Figure 1. Haplotype classification and plasma Lp(a) level of each subject. Among the 112 patients, gene analysis was performed in 105 patients in order to analyze the relationship between haplotype and plasma Lp(a) level. Plasma Lp(a) levels were significantly higher in CC than in AA, CD, and DD. The 3 SNP were classified into 4 haplotypes based on their combinations: A: G(–773), C(+93), G(+121); B: A(–773), C(+93), G(+121); C: A(–773), C(+93), A(+121); and D: A(–773), T(+93), G(+121)[28]. Each individual had 2 haplotypes: CC stands for homozygotes of type C while CD stands for heterozygotes of types C and D. Only 2 subjects had haplotype B (not shown). Horizontal bars: average of each group. *p = 0.013, **p = 0.024, ***p = 0.005 (Wilcoxon rank-sum test). †Kruskal-Wallis test.

level[21], their relationship was examined statistically. Plasma Lp(a) levels were distributed in a relatively wide range, and the mean level was high, in descending order from CC to DC to DD (statistical significance between groups, p = 0.022, Kruskal-Wallis test).

DISCUSSION

The process of Lp(a)-induced vascular damage has been reported in several *in vitro* studies. Lp(a) activates endothelium by enhancing intercellular adhesion molecule-1 expression[31], and proliferates vascular smooth muscle cells by reducing the secretion of active transforming growth factor-ß[32,33]. In addition, Lp(a) interacts with macrophages, then activates endothelium, and provokes macrophage invasion of the endothelium[34,35]. Lp(a) also competes with plasminogen for plasminogen receptor because their structures are quite similar[36], and Lp(a) then inhibits thrombolysis[37]. The mechanism of the blockage of circulation in ONF remains to be clarified; however, the processes described above would affect ONF development.

In our study, patients were classified into 2 molecular weight phenotype groups, LMW or HMW, according to the molecular weight of apo(a) isoform, and then the relationship between phenotype and ONF development was examined. LMW phenotype that contained at least one LMW isoform was found to be a risk factor for development of ONF, and among the 3 factors, i.e., MW phenotype, plasma Lp(a) level, and haplotype, only the phenotype was significantly related to ONF development (Figure 2). On the other hand, previous studies have reported the relationship between plasma Lp(a) level and MW phenotype[21,26], i.e., the plasma Lp(a) level was significantly high in the LMW group. However, in our study, ONF development was not significantly related to plasma Lp(a) level (Figure 2). This showed that another factor besides plasma Lp(a) level would interact with the effect of MW phenotype on ONF development. Marcovina, *et al*[38] identified 34 isoforms of apo(a) using a high resolution SDS-agarose gel electrophoresis method followed by immunoblotting. Hervio, *et al*[39] examined the antagonistic effect of Lp(a) to plasminogen activation *in vitro* utilizing 3 of the 34 apo(a) isoforms of MW 540 kDa, 590 kDa, and 610 kDa. They reported that 540 and 590 kDa isoforms antagonized plasminogen activation in proportion to plasma Lp(a) level, while the 610 kDa isoform

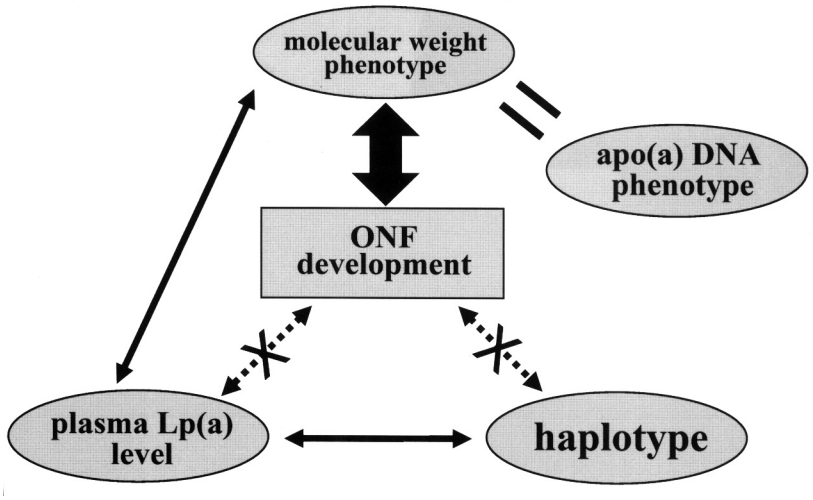

Figure 2. Molecular weight phenotype alone was significantly related to development of ONF. Solid lines show statistical significance for the relationship. Broken lines: not significant. Equal sign: There was a perfect match between the size of the apo(a) DNA phenotypes and the size of apo(a) isoforms (molecular weight phenotype) in plasma.

failed to antagonize plasminogen activation regardless of Lp(a) level. This suggested that the important factor for Lp(a) functions, e.g., the antifibrinolysis effect, is MW phenotype. MW is about 400 kDa for isoform F, 460 kDa for B, 520 kDa for S1, 580 kDa for S2, 640 kDa for S3, and 700 kDa for S4[19]. Our study demonstrated an important fact: the risk of ONF increased significantly in the LMW phenotype group that contained one of the isoforms from F to S2 in which the MW is less than 580 kDa; this finding is thought to agree with the results reported by Hervio, *et al*[39]. Therefore, we consider that LMW affects development of ONF through its inhibitory action on the fibrinolysis system.

MW isoform is a genetically determined index and it is not affected by steroid treatment. In patients who possess the LMW isoform, fibrinolysis could be inhibited at a higher degree than in patients with the HMW isoform, and thus this would induce thrombophilia more easily. Necrosis could occur because of LMW-induced thrombophilia in addition to the reported pathology of steroid-induced necrosis such as abnormal lipid metabolism[3-5] and oxidative stress *in vivo*[40].

Glueck, *et al* investigated the relationship between plasma Lp(a) level and ONF occurrence in Caucasian patients[14], and reported that plasma Lp(a) levels were significantly higher in the patients with ONF. In our study, ONF patients had high plasma Lp(a) levels, similar to findings from Glueck, *et al*, but there was not a statistically significant difference ($p = 0.056$). The plasma level in Glueck's study was 15 mg/dl in ONF patients and 5 mg/dl in the reference patients, thus the level of the ONF patients was 3 times that of the reference patients. In our study, the mean plasma Lp(a) level was 19.4 mg/dl in the ONF group and 12.9 mg/dl in the reference group, 49.2% higher in the ONF group. This difference between our findings and those of Glueck, *et al* might be explained from 2 aspects: the racial difference[41,42] and the effect of environmental factors on plasma Lp(a) levels. Examples of environmental factors are inflammatory reactions and hormones, which were not analyzed in our study. Genetic variations of apo(a) (i.e., isoform and polymorphism) are thought to determine 91% of plasma Lp(a) level, with the remaining 9% determined by environmental factors, such as inflammation and effects of estrogen and progestin[18,43,44]. Therefore, the influence of time after steroid treatment on the level of plasma Lp(a) is thought to be minor. Our findings indicate that only the isoforms have a significant influence on the risk of ONF development, and plasma Lp(a) only reaches a high level in an indirect way because the level fluctuates in relation to isoform.

In regard to the relationship between gene haplotype (integration of 3 gene polymorphisms in the promotor region) and plasma Lp(a) level, Suzuki, *et al*[21] reported that the 3 SNP in the 5' flanking region in apo(a) gene [G(–773)A, C(+93)T, and G(+121)A] affected plasma Lp(a) levels in a Japanese population, and the individuals with gene haplotype CC had significantly higher plasma Lp(a) levels than those with gene haplotype DD. We obtained the same result (Figure 1), although gene haplotype was not statistically related to development of ONF. This does not conflict with our hypothesis, i.e., MW phenotype is the factor that influences ONF development while plasma Lp(a) level has no effect on the occurrence of ONF.

In this study, the patients with the LMW phenotype were evenly divided into those who developed ONF ($n = 10$) and those who did not ($n = 14$). Patients with HMW phenotype also consisted of those with ONF ($n = 10$) and without ONF ($n = 78$). Therefore, several genetic and environmental factors besides apo(a) MW phenotype would be related to ONF

development[30]. If future studies of these factors clarified the risk of ONF development before treatment was started, corticosteroid-induced ONF could be prevented by dose adjustment and/or application of alternative therapies, not only in patients receiving transplantation of kidney, bone marrow, heart, and liver, etc., but also in those who receive treatment for collagen diseases such as systemic lupus erythematosus, asthma, and nephrotic syndrome.

We examined the relationship between corticosteroid-induced femoral head osteonecrosis after renal transplant in Japanese patients and apo(a) molecular weight phenotype, plasma Lp(a) level, and apo(a) gene polymorphism, and found that the risk of ONF is high in patients with a low molecular weight apo(a) isoform. Preoperative analysis of apo(a) molecular weight phenotype would predict postoperative development of ONF, and this analysis would assist development of tailor-made medication for patients who are scheduled to have corticosteroid treatment.

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