Study of V1a Vasopressin Receptor Gene Single Nucleotide Polymorphisms in Platelet Vasopressin Responsiveness

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There is a significant heterogeneity among individuals in terms of platelet aggregation response to arginine vasopressin (AVP). The aim of this study was to evaluate whether four single nucleotide polymorphisms (SNPs) in the promoter region of vasopressin V1a receptor gene (*V1aR*) could be used as genetic markers for divergent platelet aggregation response to AVP. Seventeen of 33 subjects showed more than 60% of maximum platelet aggregation and were classified as responders. Sixteen were classified as nonresponders because they had less than 30% aggregation. In a preliminary study, *V1aR* gene sequences were determined in two responders and two nonresponders. We found four SNPs in the promoter region of the *V1aR* gene: –6951G/A, –4112A/T, –3860T/C, and –242C/T. In all 33 subjects the genotypes of four SNPs were determined using either polymerase chain reaction (PCR) with allele-specific primers or PCR followed by restriction-fragment length polymorphism (RFLP). There were no differences in the AVP-induced aggregation between the subjects with and without variant alleles of each four SNPs. The genotype frequencies of four SNPs of *V1aR* were almost identical between AVP responders and nonresponders. These results suggest that the four SNPs in the promoter region of the *V1aR* gene may not be useful as genetic markers for platelet aggregation heterogeneity. J. Clin. Lab. Anal. 20:87–92, 2006. © 2006 Wiley-Liss, Inc.

Key words: receptors; vasopressin; V1a receptor; SNPs; platelet aggregation heterogeneity

INTRODUCTION

Platelet aggregation plays a central role in the pathogenesis of acute thrombosis in coronary heart disease (1,2), stroke, and peripheral arterial disease (3). Increased platelet aggregability is associated with hypertension, diabetes mellitus (4,5), and hypercholesterolemia (6,7). A number of vasoactive endogenous ligands for specific receptors on the platelet surface cause platelet aggregation (8). There is also evidence of a genetic influence on platelet aggregation heterogeneity (9–15). The gene variations in ligands and their receptors have been shown to have an association with aggregation heterogeneity. Therefore, these gene variations are candidates for genetic susceptibility markers of acute cardiovascular events.

Arginine vasopressin (AVP) is secreted from the posterior pituitary and acts as a vasoactive substance and an antidiuretic hormone (16). G protein-coupled V1a and V2 AVP receptors are responsible for vasopressor and antidiuretic actions, respectively. AVP has also been reported to stimulate platelet aggregation via its V1a receptor. A genetic regulation of platelet V1a receptors (V1aR) is suggested by the demonstration of a significant heterogeneity in the aggregation response to AVP (17). V1aR comprises 418 amino acids of the

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open reading frame and its gene is within 6.4 kb with two coding exons separated by a 2.2-kb intron. Two previous reports (18,19) showed that there are microsatellite motifs and single-nucleotide polymorphisms (SNPs) in the *V1aR* gene and its 5′ upstream region. However, it is yet uncertain whether *V1aR* gene polymorphism is a marker of platelet responsiveness to AVP. Therefore, in the present study we screened the *V1aR* gene polymorphisms and studied the possible association between these polymorphisms and aggregation heterogeneity to AVP.

MATERIALS AND METHODS

Study Subjects

The study included 33 young healthy volunteers (age [mean $\pm$ SD] = 22 $\pm$ 1 years). Inclusion was based on both clinical characteristics and routine laboratory tests performed at our Division of Laboratory Medicine. None of the subjects had diabetes mellitus, hypertension, hypercholesterolemia, history of smoking, or hyperlipidemia. None had any history of atherosclerotic disease, abnormal bleeding, or arterial or venous thrombotic disorders. The subjects were asked to fast overnight and to refrain from intensive exercise. The subjects did not ingest ethanol, aspirin, or other medications known to alter platelet function for at least one week prior to the study. Informed consent was obtained from each subject and the institutional ethics review committee approved the study protocol.

Sample Collection

Citrated whole fasting blood was collected from each subject for platelet aggregation tests and detection of polymorphisms in the *V1aR* gene.

Platelet Aggregation Tests

To avoid platelet activation in vivo due to physical exercise, the study subjects were at rest for at least 15–20 minutes prior to blood collection. Blood samples were collected by clean plastic syringes. Plastic tubes (TERUMO, Tokyo, Japan) containing 3.13% sodium citrate in a ratio of 1:9 were used to collect blood and the contents were immediately mixed. Platelet-rich plasma (PRP) was prepared by centrifuging at 1,000 rpm for 10 minutes. The plasma layer was examined for red cells, and special measures were taken to avoid red and white blood cells during transfer. With the use of a plastic pipette, the PRP was carefully transferred into a polystyrene plastic tube (BD Falcon, Becton Dickinson and Co., Franklin Lakes, NJ) and kept at room temperature. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 3,000 rpm for 10 minutes. The platelet counts were within 20–40 $\times$ 10^4/μl. Platelet aggregation was measured by transparency detection of the PRP sample in comparison with each relevant PPP using a platelet aggregation analyzer (MCM hematracer 313, PAM-12C; MC Medical Inc., Tokyo, Japan). The aggregation response was traced for one minute before and seven minutes after the addition of 20 μL of vehicle ADP, Arkray Inc., Kyoto, Japan, or AVP. Sigma Chemical Co., St. Louis, MO) into 200 μL of PRP. The final concentrations of ADP and AVP were 5 μM and 1 μM, respectively. The results of platelet aggregation were always interpreted against ADP as the positive control and vehicle as the negative control. Samples that showed insufficient aggregation to ADP were not included in the study. No samples showed an aggregation response to the vehicle. All reagents for the platelet aggregation were freshly reconstituted. The analysis was always performed within 30–60 minutes by the same investigator after the PPP was prepared.

Determination of Genotypes for the Four Variants of the *V1aR* Gene

Genomic DNA was extracted from whole blood using a commercial DNA extraction kit (SMI TEST; Sumitomo Bio-Medical, Kashima, Japan). Our preliminary study identified four novels SNPs when nucleotide sequences of the *V1aR* gene, including its promoter, were determined in four Japanese individuals by polymerase chain reaction (PCR) direct sequencing using the BigDye Terminator method. An automated capillary DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) was used.

In the present study we genotyped four SNPs in the promoter region of *V1aR*: –6951G/A, –4112A/T, –3860T/C, and –242C/T. The primers used to detect these four polymorphisms in the study population were designed in the laboratory. The genotype for the –4112A/T polymorphism was identified by PCR with allele-specific primers (PCR-ASA). Genotypes for the other three variants were identified by PCR followed by restriction fragment length polymorphism (PCR-RFLP). Restriction enzymes (Bfa I, New England Biolabs, Beverly, MA; Pst I, Takara Bio Inc., Otsu Shiga, Japan; and Tsp509 I, New England Biolabs) were used to determine the genotypes for –6951G/A, –242C/T, and –3860T/C, respectively. The primers used to detect the four variants are shown in Table 1.

Statistical Analysis

The data are expressed as the mean $\pm$ SD unless otherwise stated. The relationship between responses to AVP and those to ADP was evaluated using Pearson's correlation analysis. The association between

TABLE 1. Primers Used to Detect Four Polymorphisms in the Promoter Region of Vasopressin *V1a* Receptor

Variants	Orientation	Location	Sequence
−6951 G/A	Forward	1714–1733	5′-TGT TGC TTC AAA CTT GGC AG-3′
	Reverse	2424–2400	5′-TGT CTA AAT TTG ATC ATC AAG ACT C-3′
−4112 A/T	Forward-w	4766–4781	5′-GAA GCG GGA GGT TTC A-3′
	Forward-m	4766–4781	5′-GAA GCG GGA GGT TTC T-3′
	Reverse	4909–4890	5′-AGA TTC ACT GAG CCA GAC TA-3′
−3860 T/C	Forward	4873-4899	5′-AAT AGT CAC TTA TGC CTT AGT CTG GCT-3′
	Reverse	5069–5041	5′-ACT CTT AGT CTG TAA CAG AGA ATG CTC CT-3′
−242 C/T	Forward	8518–8539	5′-GGA GTA GGC AAC CAG CAG TCT T-3′
	Reverse	8675–8652	5′-CCT GCT CTG CCT TTT TTC AAC TGC-3′

V1aR, vasopressin V1a receptor gene; Forward-w, forward-wild; Forward-m, Forward-mutant.

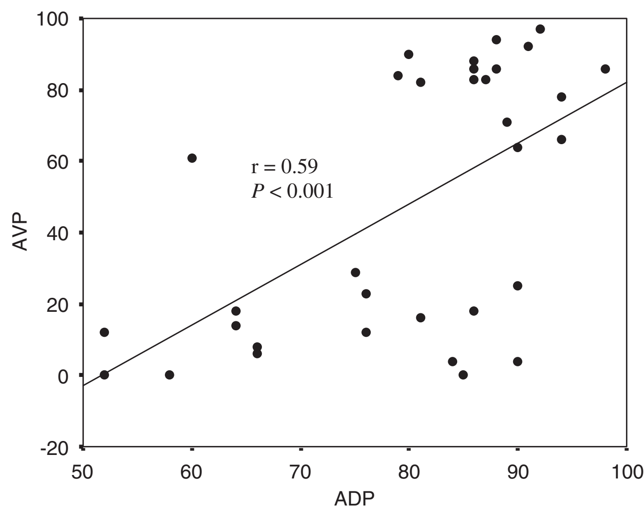

Fig. 1. Platelet aggregation in response to ADP and AVP.

gene polymorphisms and platelet response was evaluated using Fisher's exact test. The difference in platelet responses between groups was evaluated by one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Platelet Aggregation Tests

The maximum aggregation responses to AVP varied from 0% to 97% with a mean value of 47.88% ± 36.85%, whereas those to ADP varied from 52% to 98% with mean value of 79.82% ± 12.67%. Seventeen of 33 subjects showed more than 60% of maximum platelet aggregation by AVP and were categorized as responders to AVP, whereas 16 were classified as nonresponders because they had less than 30% aggregation. There was a significant correlation (r = 0.59; $P<0.001$) between responses to AVP and those to ADP (Fig. 1).

TABLE 2. Distribution of Four Variants of *V1aR* Genotypes and Allele Frequencies in 33 Healthy Japanese Individuals[a]

Variants	Number (%)
−6951 G/A	
Genotypes	
GG	23 (69.7)
GA+AA	10 (30.3)
Alleles	
G	55 (83.3)
A	11 (16.7)
−4112 A/T	
Genotypes	
AA	27 (81.8)
AT+TT	6 (18.2)
Alleles	
A	59 (89.4)
T	7 (10.6)
−3860 T/C	
Genotypes	
TT	14 (42.4)
TC+CC	19 (57.6)
Alleles	
T	41 (62.1)
C	25 (37.9)
−242 C/T	
Genotypes	
CC	28 (84.8)
CT+TT	5 (15.2)
Alleles	
C	60 (90.9)
T	6 (9.1)

[a]Percentages are over column total.

Genotypes and Aggregation Heterogeneity

The genotypes and allele frequencies of the –6951G/A, –4112A/T, –3860T/C, and –242C/T variants are shown in Table 2.

When platelet aggregations in response to AVP were compared among the groups defined by each of the four

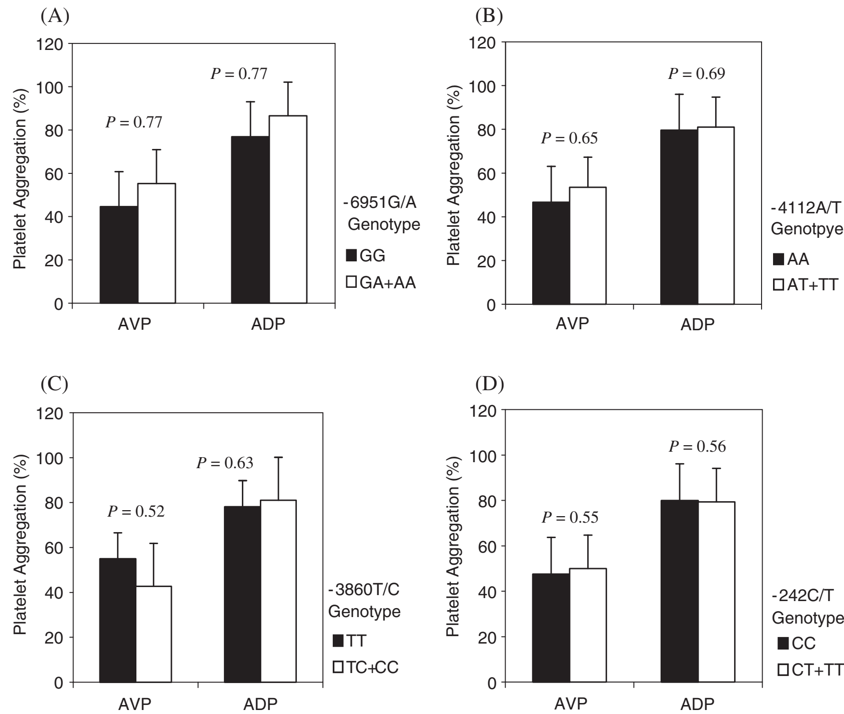

Fig. 2. Platelet aggregation in response to AVP and ADP among genotypes defined by – 6951G/A (**A**), –4112A/T (**B**), –3860T/C (**C**), and –242C/T (**D**). Values of platelet response are given as the mean ± SEM.

variants of *V1aR*, no significant association was found between the genotype and the platelet response ($P = 0.77$ for –6951G/A, 0.65 for –4112A/T, 0.52 for –3860T/C, and 0.55 for –242C/T). The aggregation response to ADP also showed no significance ($P = 0.77$ for –6951G/A, 0.69 for –4112A/T, 0.63 for –3860T/C, and 0.56 for –242C/T; Fig. 2).

We compared genotype distribution of four variants in *V1aR* between the responder and nonresponder groups. However, there was no significant association ($P = 0.52$ for –6951G/A, 0.32 for –4112A/T, 0.57 for –3860T/C, and 0.68 for –242C/T; Table 3).

TABLE 3. Genotypes of Four Variants of *V1aR* in Arginine Vasopressin (AVP) Responder and Nonresponder Groups

Variants of *V1aR*[a]	AVP Responder (n = 17)	AVP Nonresponder (n = 16)	*P*
−6951G/A			
GG, n (%)	11 (64.7)	12 (75.0)	0.52
GA+AA, n (%)	6 (35.3)	4 (25.0)	
−4112A/T			
AA, n (%)	13 (76.5)	14 (87.5)	0.32
AT+TT, n (%)	4 (23.5)	2 (12.5)	
−3860T/C			
TT, n (%)	8 (47.1)	6 (37.5)	0.57
TC+CC, n (%)	9 (52.9)	10 (62.5)	
−242C/T			
CC, n (%)	14 (82.4)	14 (87.5)	0.68
CT+TT, n (%)	3 (17.6)	2 (12.5)	

[a]Percentages are over column total.
AVP, arginine vasopressin; *V1aR*, vasopressin V1a receptor gene.

DISCUSSION

In the present study we confirmed that platelet aggregation response to AVP varies among individuals. We also found a significant correlation between platelet aggregation response to ADP and AVP. In a preliminary study we found four novel SNPs in the promoter region by sequencing the *V1aR* gene and its promoter in four Japanese individuals who showed different platelet responses to AVP. None of these SNPs were previously reported. However, we failed to obtain evidence that these *V1aR* gene SNPs influence heterogeneity in platelet aggregation response to AVP. These results indicate that *V1aR* gene polymorphisms may not play a role in aggregation heterogeneity. It is possible that variations other than those examined in the present study may be useful DNA markers for detecting a possible link between genotypes and phenotypes.

However, it also appears that genes involved in the post-receptor signal pathway, such as protein kinase C (16), may play a dominant role in the diversity of AVP action on platelets.

The present study is the first to compare platelet aggregation in response to ADP and AVP. The significant correlation between these two agonists suggests that they may share a common pathway in inducing platelet aggregation. Birnbaumer (20) noted that AVP action through the V1a receptor is mediated by activating phospholipase C, via Gq/11. Jin and Kunapuli (21) demonstrated that coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation (P2Y1 activates phospholipase C via Gq, and P2Y12 inhibits adenylyl cyclase via Gi). In another study, platelets from knock-out mice lacking the gene coding for the αq subunit of the Gq protein did not aggregate in response to ADP (22), indicating that the activation of phospholipase C is essential for platelet aggregation. Therefore, both ADP and AVP require binding to Gq protein to activate phospholipase C in inducing platelet aggregation.

The present study is the first to investigate the implication of *V1aR* gene polymorphisms in recognizing individuals who have enhanced platelet activation by AVP and consequently are at risk for cardiovascular disease. Platelet aggregation plays a central role in the pathogenesis of acute thrombosis in cardiovascular events. Gene polymorphisms play a significant role as markers in association and linkage studies. Therefore, identification of genetic markers of platelet hyper-responsiveness would be beneficial for developing anti-platelet therapies to reduce cardiovascular risk.

Platelet aggregation heterogeneity is thought to be under genetic influence. However, studies that examined the genetic epidemiology of abnormal platelet aggregability failed to provide strong evidence for specific molecular variants that increase aggregability in cardiovascular diseases (9–15). Both the GPIIIa genotype and the fibrinogen Hind III β-148 genotype make only a small contribution to platelet aggregation (23).

Although numerous variants of the AVP V2 receptor gene have been found in patients with nephrogenic diabetes insipidus (18), there is not much information regarding mutations or polymorphisms in the AVP *V1aR* gene. Thibonnier et al. (18) revealed several DNA microsatellite motifs by sequencing the *V1aR* gene and its 5′ upstream region. Recently Saito et al. (19) found 20 variations in the *V1aR* gene in a Japanese population. None of these variations were identified in our screening study. *V1aR* gene variations are useful DNA markers for investigating the possible correlation between genotypes and phenotypes. In a study of human hypertension, Thibonnier et al. (18) reported that the distributions of the *V1aR* microsatellite polymorphisms did not differ between hypertensives and normotensives. In rodents, however, Young et al. (24) found that promoter variation of the *V1aR* is the key to determining affiliative behaviors. They also found altered V1aR expression levels within the central nervous system in accordance with the variation.

In conclusion, the current results suggest that ADP and AVP may share a common pathway in inducing platelet aggregation, and four SNPs (–6951G/A, –4112A/T, –3860T/C, and –242C/T) in the promoter region of the *V1aR* gene may not be useful as genetic markers of platelet aggregation heterogeneity.

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