Table 3. Clinical Profiles of Hypertensives with Missense Mutations in Exon13 of *SCNN1B*

	Case					
	1	2	3	4	5	6
Polymorphism	Pro592Ser	Pro592Ser	Pro592Ser	Thr594Met	Thr594Met	Glu632Lys
Age (years old)	62	73	67	58	91	66
Sex	male	male	female	female	female	female
BMI (kg/m^2)	28.2	26.3	22.8	28.5	25.6	20.5
Diagnosis	EHT, NIDDM, HL, OMI	EHT, HL, EA	EHT, NIDDM, HL	EHT, NIDDM, HL	EHT, HL, OCI	RVHT, IGT, HL, ASO, OCI
HT duration (years)	30	20	11	23	50	11
HT family Hx	father	mother	none	brother	mother, brother	none
SBP (mmHg)	140	138	150	174	122	150
DBP (mmHg)	88	70	80	96	62	70
Medication	CCB, BB, spironolactone	CCB	CCB	CCB, ARB, AB	CCB, BB	CCB, ARB, spironolactone
Na (mEq/l)	137	138	139	140	142	142
K (mEq/l)	4.3	4.9	3.9	4.4	4.4	2.7
Cl (mEq/l)	101	109	100	102	108	98
Creatinine (mg/dl)	0.8	0.9	0.3	0.5	0.9	1.2
Overt proteinuria	−	−	−	+	−	+
PRA (ng/ml/h)	4.8	no data	1.1	0.8	1.4	22
PAC (ng/dl)	1.8	no data	8	22.6	9.3	102.9
FBS (mg/dl)	109	89	143	110	108	106
HbA1c (mg/dl)	7.2	5.3	8.0	5.3	5.6	5.7

SCNN1B, β-subunit of epithelial sodium channel gene; BMI, body mass index; EHT, essential hypertension; NIDDM, non-insulin dependent diabetes mellitus; HL, hyperlipidemia; OMI, old myocardial infarction; EA, effort angina; OCI, old cerebral infarction; RVHT, renovascular hypertension; IGT, impaired glucose tolerance; ASO, atherosclerotic obliterance; HT, hypertension; Hx, history; SBP, systolic blood pressure; DBP, diastolic blood pressure; CCB, calcium channel blocker; BB, β-adrenergic blocker; ARB, angiotensin II receptor blockade; AB, α1-adrenergic blocker; PRA, plasma renin activity; PAC, plasma aldosterone concentration; FBS, fasting blood sugar.

SCNN1B (cases 2, 3, 5, Table 3) and *SCNN1G* (cases 1–3, Table 4) did not show specific abnormalities of electrolytes, renin and aldosterone levels, or renal function.

Discussion

By the direct DNA sequence method, we determined the DNA sequence of the 381 bp-coding region in exon 13 of the *SCNN1B* gene and of the 381 bp-coding region in exon 12 of the *SCNN1G* gene in 948 and 953 Japanese patients with hypertension and identified seven and five mutations, respectively. Several previous studies have screened for mutation in exon 13 of the *SCNN1B* gene using the single-strand conformational polymorphism (SSCP) method (*11–13*, *15*, *16*). SSCP is highly sensitive for detecting polymorphism (*25*). We adopted the direct DNA sequence method instead of the SSCP method, because it is more accurate than the SSCP method. A screening of mutations by sequencing was previously performed in a large Japanese cohort including 90 hypertensives and 51 controls (*24*). In that study, however, the authors only sequenced a part of *SCNN1B* and none of *SCNN1G*. Therefore, our study is the first complete, large-scale screening effort to detect mutations in the coding region of exon 13 of the *SCNN1B* gene and exon 12 of the *SCNN1G* gene, *i.e.*, in the regions where the causative mutations of Liddle's syndrome are located.

Among the three missense mutations we identified in exon 13 of the *SCNN1B* gene, the P592S mutation was previously identified in four out of 803 Japanese subjects by SSCP screening followed by DNA sequencing (*13*). It has been reported that this missense mutation was not associated with either home or casual blood pressure values. Although we were not able to determine whether this P592S mutation in the *SCNN1B* gene was associated with blood pressure elevation in the present study, since all our subjects were hypertensive, one essential hypertensive patient with the P592S mutation showed high PRA, low PAC, and normokalemia, even though he was taking β blockade and spironolactone.

The T594M mutation we identified in the *SCNN1B* gene was originally reported in 6.1% of African-American subjects but not seen in Caucasians (*11*). In other studies, this mutation was not observed in Japanese individuals (*13*, *14*). So far, therefore, the T594M mutation has been identified only in individuals of African descent (*11–15*). However, our intensive sequence study clearly showed that this mutation is also present in the Japanese population, even though

Table 4. Clinical Profiles of Hypertensives with Missense Mutations in Exon12 of *SCNN1G*

	Case		
	1	2	3
Polymorphism	Ala578Val	Pro603Ser	Leu609Phe
Age (years old)	70	80	65
Sex	female	male	male
BMI (kg/m^2)	21.5	25.5	24.4
Diagnosis	EHT, NIDDM	EHT, NIDDM, HL	EHT, EA
HT duration (years)	30	32	7
HT family Hx	none	mother	none
SBP (mmHg)	134	160	128
DBP (mmHg)	74	110	90
Medication	CCB, ACEI, ARB	CCB	—
Na (mEq/l)	143	140	140
K (mEq/l)	3.8	4.2	4.3
Cl (mEq/l)	106	105	106
Creatinine (mg/dl)	0.5	1.2	1.0
Overt proteinuria	—	+	—
PRA (ng/ml/h)	0.3	1.3	2.8
PAC (ng/dl)	12.2	9.8	15.6
FBS (mg/dl)	136	105	93
HbA1c (mg/dl)	8.6	6.1	4.6

SCNN1G, γ-subunit of epithelial sodium channel gene; BMI, body mass index; EHT, essential hypertension; NIDDM, non-insulin dependent diabetes mellitus; HL, hyperlipidemia; EA, effort angina; HT, hypertension; Hx, history; SBP, systolic blood pressure; DBP, diastolic blood pressure; CCB, calcium channel blocker; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blockade; PRA, plasma renin activity; PAC, plasma aldosterone concentration; FBS, fasting blood sugar.

the prevalence is quite low (2/956, 0.2%). In a study on black residents in London, a gender difference was observed, with females tending to carry the M allele (*18*). Notably, two individuals having the M allele in our study were also females.

Whole-cell voltage clamp recording of lymphocytes from patients having the variant channel with the T594M mutation showed a greater response following stimulation with cAMP analog compared to wild-type lymphocytes (*11*). Furthermore, the cells isolated from the homozygote showed that phorbol 12-myristate 13-acetate had no effect on cAMP analog-induced electrophysiological responses, indicating that the T594M mutation plays an important role in regulating ENaC (*17*). These functional analyses may explain the clinical phenotype of patients with the T594M mutation. The T594M mutation contributes to the elevation of blood pressure and suggests that consideration should be given to the use of amiloride in affected individuals (*19*).

In the present study, we found a novel missense mutation, E632K, in exon13 of the *SCNN1B* gene. As shown in Table 3, a patient with this missense mutation (case 6) was diagnosed with renovascular hypertension, and showed specific abnormalities in potassium level, PRA and PAC. This patient had taken angiotensin II receptor antagonist and spironolactone, but still had marked hypokalemia. Therefore, to clarify the consequences of this mutation, functional analysis of the mutant ENaC β subunit with E632K will be needed.

In previous studies, individuals with either one of two synonymous mutations, Ser520 and Thr594, in exon 13 of the *SCNN1B* gene showed no significant differences in home or casual blood pressure values, age, body mass index, biochemical profiles of electrolytes or PRA, gender, or use of antihypertensive medication compared to individuals without mutation (*13*, *16*). In the present study, we confirmed that these two mutations did not influence body mass index, biochemical profiles, or use of antihypertensive medications. However, we were not able to evaluate these mutations with respect to blood pressure elevation, since all our subjects were hypertensives.

Three hypertensive patients with novel missense mutations in exon 12 of the *SCNN1G* gene did not have specific abnormalities in electrolytes or the renin-angiotensin aldosterone system. However, it was considered worthwhile to perform functional analysis of these missense mutations in the *SCNN1G*, because single nucleotide polymorphism in the promoter region has been associated with blood pressure regulation in Japanese (*21*).

In summary, we identified three missense mutations, including one novel mutation, E632K, in the *SCNN1B* gene and three novel missense mutations, A578V, P603S and L609F, in the *SCNN1G* gene, in Japanese patients with hy-

pertension.

It remains unclear what roles these missense mutations of the *SCNN1B* or *SCNN1G* gene play in the pathogenesis of hypertension and the regulation of electrolytes in hypertensive patients. Thus, further investigations, including functional analyses, of these mutations will be needed.

Acknowledgements

We are grateful to Dr. Mariko Banno, Ms. Yoko Tokunaga and Chiyako Imai for their excellent technical assistance.

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reported to be associated with the incidence of MI [3]. However, a second report showed that this association was valid in females, but not in males [5]. We also did not observe any positive association in our male subjects. Thus, in Japanese male subjects, *MMP-3* 5A/6A does not seem to predict the incidence of MI. In females, it is possible that we did not observe any positive association, at least partially, due to the relatively small number of female patients with MI, and further investigations may be needed.

Several association studies and meta-analyses have investigated the association between the *MTHFR* gene and an increased risk of MI [6]. In the Japanese population, Yamada *et al.* did not find an association between *MTHFR* C677T and the incidence of MI [5]. Considering these and our present results, it is unlikely that *MTHFR* C677T is associated with an increased risk of MI in Japanese.

Acknowledgements

This study was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese

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To cite this article: Sakata T, Okamoto A, Mannami T, Matsuo H, Miyata T. Protein C and antithrombin deficiency are important risk factors for deep vein thrombosis in Japanese. *J Thromb Haemost* 2004; **2**: 528–30.

The frequency of factor (F)V Leiden mutation is relatively high among individuals of Caucasian descent, being from 2 to 15% in the general population and up to 50% in selected patients with thromboembolism [1]. The risk of the first episodes of thromboembolism as estimated in a large case–control study is 7-fold for heterozygous FV Leiden carriers [2]. Although the frequency of deficiencies of natural anticoagulants, protein C or antithrombin in the general population is low, prospective studies indicate that low levels of protein C and antithrombin

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Received 6 November 2003, accepted 6 November 2003

are associated with increased incidence of venous thrombosis [3,4]. We tried to elucidate the association between the deficiency of protein C or antithrombin and deep vein thrombosis in Japanese, since the FV Leiden is not in the Japanese population [5] and there have been no reports assessing the prevalence of protein C or antithrombin deficiency in Asian populations.

The reference group was a population randomly selected from the residents of Suita, a city located in the second largest urban area in Japan (Osaka area). The sample comprised 12 200 men and women aged 32–89 years. The subjects have been visiting the National Cardiovascular Center every 2 years since 1989 for regular health checkups [6]. In the present study, 4517 blood donors (2090 male, 2427 female) aged 32–89 years were enrolled for measurement of protein C and antithrombin activity. As a study population for the patient group, 108 consecutive outpatients between April 1994 and March 1998 (54 men, 54 women; mean age ± SD 57.8 ± 17.2 years and 49.6 ± 18.0 years, respectively) with deep vein thrombosis admitted to the Department of Cardiology at the National Cardiovascular Center were enrolled. The diagnoses of deep vein thrombosis were based on radioisotope venography and/or contrast venography. Protein C amidolytic activity was measured using S-2366 as a substrate and Protac derived from *Agkistrodon contortrix* venom as the activator. Antithrombin activity was measured as a heparin cofactor activity using a chromogenic substrate S-2238.

In the identification of protein C deficiency, the ratio of protein C to factor (F)X was recommended by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [7]. In our study, we calculated the ratio of protein C activity to antithrombin activity (AT/PC ratio), because the antithrombin activity assay was easier and more reliable than the FX antigen assay. Using the criteria of an AT/PC ratio > 3 SD (1.27) and protein C activity < 3 SD (59.3%), six and seven individuals were identified as a heterozygous protein C deficiency from the reference group and patient group, respectively. Furthermore, by the same criteria, seven and six individuals were identified as an antithrombin deficiency from the reference group and patient group, respectively (Table 1).

The prevalence of protein C deficiency (0.13%) obtained by use of the combined criteria of protein C activity and the antithrombin/protein C ratio agreed with the results previously reported in Scotland (0.20%) [8]. Also, the prevalence (0.15%) of antithrombin deficiency obtained in our study was very similar to the result previously reported in Scotland (0.17%) [9].

We obtained the prevalence of protein C (6.5%) and antithrombin (5.6%) deficiencies in patients with deep vein thrombosis. The prevalence in the deep vein thrombosis group was statistically higher than that in the general population, indicating that each deficiency is a severe risk factor for deep vein thrombosis in the Japanese population (Table 1). The prevalence of protein C deficiency in the deep vein thrombosis group in Europe or the USA was reported to be 1.1% and 2.4% in unselected and selected patients, respectively, and antithrombin deficiency was 3.2% and 3.8% in unselected and selected patients, respectively [10]. Therefore, the prevalence of each deficiency in the Japanese deep vein thrombosis group seemed to be higher than that in Westerners with deep vein thrombosis. One possible reason is that the FV Leiden mutation and the prothrombin G20210A mutation are present only in the Caucasian population [1,11] and not in the Japanese population [5,12].

Table 1 Comparison of prevalence of protein C and antithrombin deficiencies between the deep vein thrombosis group and the general population

	Number of heterozygote (prevalence, %)			
	Patients with DVT ($n = 108$)	General population ($n = 4517$)	Odds ratio (95% CI) (vs. general population)	*P*-value
Protein C	7 (6.48%)	6 (0.13%)	52.1 (17.2, 157.9)	<0.0001
Antithrombin	6 (5.56%)	7 (0.15%)	37.9 (12.5, 114.8)	≤0.0001

CI, Confidence interval; DVT, deep vein thrombosis.

Acknowledgements

We would like to express our gratitude to O. Hishikawa, the President, and K. Kawanishi, a committee in chief of Suita City Medical Association, for their continuous support of our population survey in this area. We also express our gratitude to S. Baba and H. Tomoike for sample collection, steering and discussion. This study was supported by Health Sciences Research Grants for Research on Specific Diseases, Blood Coagulation Disorders from the Ministry of Health Labor and Welfare, Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research (of Japan), and Special Coordination Funds for Promoting Science and Technology, the Japanese Government.

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Lethal toxin of *Bacillus anthracis* inhibits tissue factor expression in vascular cells

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To cite this article: Rao LVM, Ngyuen M, Pendurthi UR. Lethal toxin of *Bacillus anthracis* inhibits tissue factor expression in vascular cells. *J Thromb Haemost* 2004; **2**: 530–32.

Bacillus anthracis, the causative agent of anthrax, has been postulated to be a likely agent of biowarfare and bioterrorism, and received much notoriety following a recent outbreak of anthrax associated with bioterrorism in the USA [1]. Case reports of patients with anthrax attacks of 2001 document that many of these patients developed disseminated intravascular coagulation (DIC), respiratory failure and septic shock, in some cases leading to death [2–5]. The clinical course in patients with fatal inhalational anthrax [4] and cutaneous anthrax [2,6] showed convincing evidence of the activation of coagulation upon anthrax infection. At present, it is unclear how anthrax activates the coagulation system.

Three proteins secreted by *B. anthracis*, lethal factor (LF), edema factor (EF) and protective antigen (PA), are central to its pathogenicity [7]. Lethal toxin (LeTx), a complex of LF and PA, is primarily responsible for anthrax virulence [7]. Sublytic concentrations of LeTx were shown to induce interleukin (IL)-1β and tumor necrosis factor (TNF)-α in macrophages *in vitro* [8]. Further, the administration of LeTx to mice was shown to induce endotoxic shock-like death, and antisera against IL-1β and TNF-α protected mice from LeTx challenge [8,9]. Since the septic shock caused by *B. anthracis*/purified LeTx resembles *Escherichia coli*/lipopolysaccharide (LPS)-mediated shock, where tissue factor (TF) is shown to play a critical role in the pathogenesis of septic shock [10–12], we investigated in the present study whether LeTx, similar to LPS, induces TF expression in vascular cells.

To determine whether anthrax toxins induce TF expression in vascular cells, monolayers of human umbilical vein endothelial cells (HUVEC) were treated with varying concentrations of LeTx or edema toxin (EdTx) (10 ng to 1000 ng mL^{-1}) for 6 h and the induction of TF activity was measured in factor X activation assay [13]. Both LeTx and EdTx failed to induce TF activity in endothelial cells (Fig. 1A). Similarly, a combination of LeTx and EdTx treatment also had no effect on TF induction. When HUVEC were stimulated with LPS, as expected, there was a marked increase in TF activity. Interestingly, pretreatment of endothelial cells with LeTx markedly attenuated LPS-induced TF expression (Fig. 1A). The suppressive effect of LeTx was dose-dependent and 100 ng mL^{-1} LeTx suppressed TF induction maximally. In contrast to LeTx, EdTx had only a minor effect on LPS-induced TF activity in HUVEC. As observed with endothelial cells, LeTx also markedly inhibited LPS-induced TF expression in freshly isolated human mononuclear cells

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Received 28 October 2003, accepted 31 October 2003

Original Article

Evaluation of the Lys198Asn and −134delA Genetic Polymorphisms of the Endothelin-1 Gene

Chihiro TANAKA, Kei KAMIDE*, Shin TAKIUCHI*, Yuhei KAWANO*, and Toshiyuki MIYATA

Endothelin-1 (ET-1) is a potent vasoconstrictor and shows various pharmacological responses. Two single nucleotide polymorphisms in the ET-1 gene (*EDN1*) have been reported to be associated with blood pressure (BP). One is the Lys198Asn polymorphism, which showed a positive association with BP in overweight people. Another is the 3A/4A polymorphism (−134delA) located in the 5′-untranslated region. In this study, we investigated the expression of the Lys198Asn polymorphism in ET-1 *in vitro*, as well as the association between either of the two polymorphisms and the plasma ET-1 level. We expressed both the major (Lys-type) and minor type (Asn-type) preproET-1 in three different cell lines, and measured the levels of ET-1 and big ET-1 in the culture supernatant. There was no significant difference in the levels of ET-1 or big ET-1 between the Asn-type and Lys-type transfectant. In the association study, the plasma levels of ET-1 in 54 hypertensive patients having an amino acid substitution from Lys to Asn at position 198 were not different from those of hypertensives without the substitution. However, we found a significant difference in ET-1 levels between individuals with the 3A/3A and 3A/4A genotypes. Our transient expression study indicates that the Lys198Asn polymorphism may not directly affect ET-1 and big ET-1 production. Another variant in the *EDN1* gene in linkage disequilibrium with the Lys198Asn polymorphism may be responsible for the association with BP, or the interaction between the *EDN1* Lys198Asn polymorphism and other factors such as obesity may be involved in the mechanisms elevating BP *in vivo*. (*Hypertens Res* 2004; 27: 367–371)

***Key Words*: endothelin-1, Lys198Asn polymorphism, hypertension, vasoactive peptide, plasma endothelin-1 level**

Introduction

Endothelin-1 (ET-1), a 21-amino-acid peptide, is a potent vasoconstrictor and pressor substance mainly produced by vascular endothelial cells (*1*). ET-1 is thought to contribute to the development of cardiovascular diseases, and is assumed to modulate vascular tone and blood flow and promote vascular cell growth in an autocrine or paracrine fashion through two subtypes of receptor (*2*). In a previous clinical study, the plasma ET-1 level was significantly higher in essential hypertensive patients (*3*). Furthermore, an elevated ET-1 level has been associated with carotid atherosclerosis and asymptomatic cerebrovascular lesions in patients with essential hypertension (*4*). Thus, it is thought that ET-1 plays an important role in the etiology of hypertension and atherosclerosis (*5*).

ET-1 is synthesized from a 212-amino-acid precursor protein, preproET-1, through multiple proteolytic steps, as shown in Fig. 1 (*2*, *6*, *7*). In the first step, preproET-1 is cleaved by signal peptidase, resulting in the formation of proET-1. ProET-1 is then cleaved at the paired dibasic amino acids by a furin-like enzyme to give rise to 38-amino-acid big ET-1 or other intermediates. Big ET-1 is subsequently cleaved at Trp73-Val74 by another endopeptidase, endothelin converting enzyme (ECE), resulting in the production of mature ET-1. Although ET-1 is biologically active, intermediate polypeptides, including big ET-1, are bio-

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This study was supported by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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Received December 8, 2003; Accepted in revised form February 4, 2004.

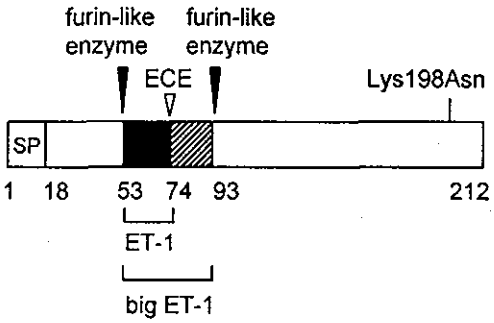

Fig. 1. *Processing of preproendothelin-1. The cleavage sites of furin-like enzyme and endothelin converting enzyme are shown by black and white arrowheads, respectively. ET-1 is a 21-amino acid peptide with residues 53–73. Big ET-1 is a 40-amino acid peptide with residues 53–92. The position of the Lys198Asn polymorphism is also shown by an arrow. SP, signal peptide; ET-1, endothelin-1; ECE, endothelin converting enzyme.*

logically inactive (*8*).

There are two single nucleotide polymorphisms in the ET-1 gene (*EDN1*) that are associated with blood pressure (BP). A G-to-T transversion in exon 5 that causes the Lys-to-Asn substitution at codon 198 has been reported (*9–11*). Recent association studies suggest that the Lys198Asn polymorphism interacts with BP in overweight people (*9*, *10*), or with body mass index (BMI) in association with hypertension (*11*). Concerning plasma ET-1 level, pregnant women with the Asn/Asn genotype have significantly elevated plasma ET-1 levels, and the Asn-allele has been associated with raised systolic BP (SBP), suggesting the functional importance of this polymorphism in pregnancy (*12*). In a study using human mammary artery rings *in vitro*, artery segments from individuals with the Lys/Lys genotype were found to have an elevated response to phenylephrine-induced contractions compared to those with the Lys/Asn or Asn/Asn genotype (*13*).

Another polymorphism associated with hypertension is the 3A/4A variant (−134delA) located 138 bp downstream from the transcription start site in the 5′-untranslated region in *EDN1* (*14*, *15*). In an *in vitro* assay for using the luciferase reporter gene, Popowski *et al.* reported that the 4A allele showed increased expression compared to the 3A allele (*16*). Furthermore, the human umbilical vein endothelial cells (HUVECs) obtained from homozygotes with the 4A allele showed a significantly increased expression of ET-1 protein due to enhanced mRNA stability (*16*). However, the association of plasma ET-1 levels with the 3A/4A genotypes has not been investigated.

The studies on the Lys198Asn polymorphism so far performed (*9–13*) have raised the question of whether the Lys198Asn polymorphism by itself has functional significance or, alternatively, whether it is linked to another variant in the *EDN1* gene. The Lys198Asn polymorphism is located near the carboxyl terminal region, which is removed from preproET-1 by the proteolytic action of the furin-like enzyme (Fig. 1). If the *EDN1* Lys198Asn polymorphism is directly involved in the ET-1 function, it may affect the processing of preproET-1 to mature ET-1 through big ET-1 formation. To test this hypothesis, we performed transfection experiments. We measured the concentration of ET-1 and big ET-1 in the culture supernatant obtained from Lys-type or Asn-type transfectants using three different cell lines. In addition, we measured the plasma levels of ET-1 and investigated the influence of two *EDN1* polymorphisms, Lys198Asn and 3A/4A, on the plasma ET-1 level in patients with hypertension.

Methods

Cell Culture

COS1 cells and 293 cells were purchased from American Type Culture Collection (Manassas, USA), and HUVECs were purchased from CellSystems (St. Katharinen, Germany). COS1 and 293 cells were cultured in DMEM medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, USA), while HUVECs were cultured on a type I collagen-coated culture dish in MCDB131 medium (Invitrogen) supplemented with 10 mmol/l GlutaMAX (Invitrogen), 20 mmol/l HEPES (Invitrogen), 2% FBS, and 10 ng/ml basic fibroblast growth factor (Roche Diagnostics, Mannheim, Germany). These cells were incubated in humidified 5% CO_2 in air at 37°C. HUVECs were used for the experiments after 3–5 passages.

Site-Directed Mutagenesis

The preproendothelin-1 cDNA covering the entire coding region was subcloned between the *Eco*RI and *Sal*I sites of the pCI mammalian expression vector (Promega, Madison, USA). Since this cDNA encodes Lys at codon 198, the obtained expression vector was designated pCI/hET-1/Lys. To replace Lys with Asn at codon 198, site-directed mutagenesis was performed. The following primers were used for polymerase chain reaction with a *Pfu* DNA polymerase (Stratagene, La Jolla, USA) (the mutated codon is underlined): forward 5′-CAAGCTGAAAGGCAATCCCTCCAGAGAGCG-3′, and reverse 5′-CGCTCTCTGGAGGGATTGCCTTTCAGCTTG-3′. Successful introduction of this mutation was confirmed by sequencing the entire coding region, and the obtained plasmid was designated pCI/hET-1/Asn.

Transient Expression and Measurement of the Concentration of Extracellular ET-1

COS1 cells were seeded at 1×10^5 cells/well in six-well plates on the day before transfection, and transfected with a mixture of 1 μg of expression plasmid (pCI/hET-1/Lys, pCI/hET-1/Asn or pCI) and 5 ng of pMI-SEAP (Roche

Diagnostics) using 5 μl of Polyfect Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After a 24-h incubation, the culture medium was exchanged to fresh medium containing 10% FBS. The transfected cells were incubated for another 24 h and then the culture supernatant was collected for measurement of immunoreactive ET-1 and big ET-1. The activity of secreted human placental alkaline phosphatase (SEAP) in the culture supernatant was measured by SEAP Reporter Gene Assay (Roche Diagnostics) for normalization of the transfection efficiency. The 293 cells were seeded at 2×10^5cells/well in six-well plates and transfected by the same method as used for the COS1 cells, except for the use of 3 μl of FuGENE 6 Transfection Reagent (Roche Diagnostics). HUVECs were seeded at 1×10^5cells/well in six-well plates and transfected with a mixture of 0.5 μg of expression plasmid and 5 ng of pMI-SEAP using 3 μl of FuGENE 6 Transfection Reagent. After a 6-h incubation, the culture medium was exchanged with fresh medium. The transfected cells were incubated for another 42 h, and then the culture supernatant was collected. The amounts of immunoreactive ET-1 and big ET-1 in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) using the Endothelin-1 Measurement Kit-IBL and Human Big Endothelin-1 Measurement Kit-IBL, respectively (Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan).

Comparison of the Plasma ET-1 Level between Each Genotype in *EDN1* Gene Polymorphisms

We measured the plasma ET-1 level and genotyped two *EDN1* gene polymorphisms, Lys198Asn (5727G>T) in exon 5 and 3A/4A in the 5′-untranslated region (−134delA), in 54 randomly selected essential hypertensive patients enrolled at the Division of Hypertension and Nephrology at the National Cardiovascular Center. All subjects provided written informed consent to participate in the study, and the study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. The plasma ET-1 level was measured by radioimmunoassay after extraction (*17*). Genotyping was performed by the TaqMan PCR method using the following probes and primers: for Lys198Asn, probes Fam-AGGCAAGCCCTCCA-MGB (Lys-allele), Vic-AAGG CAATCCCTCCAG-MGB (Asn-allele), and primers 5′-CAG CGTCAAATCATCTTTTCATG-3′ and 5′-GTCACCAATG TGCTCGGTTGT-3′, and for 3A/4A, probes Fam-AGTGCC CTTTTAACGG-MGB (4A-allele) and Vic-AAGTGCCC TTTAACGG-MGB (3A-allele), and primers 5′-AAACAGC TTCAGGTCCCTCAAA-3′ and 5′-GCAGTCCCAGCTCTC CACC-3′ (*18*).

Statistical Analysis

The comparison of ET-1 and big ET-1 levels in the culture supernatant between the different transfected cell lines, and of the plasma ET-1 level and all of the clinical parameters between the two genotypes in patients with essential hypertension were performed by unpaired Student's *t*-test using the program StatView (SAS Institute, Inc., Cary, USA). A value of $p<0.05$ was considered to be statistically significant. In order to evaluate the linkage disequilibrium (LD), we calculated the D' and r^2 value using SNPAlyze version 3.0 software (DYNACOM, Mobara, Japan).

Results

The Lys198Asn Polymorphism Did Not Affect the ET-1 and Big ET-1 Levels in the Culture Supernatant

To examine whether the Lys198Asn polymorphism in the *EDN1* gene affects production of ET-1, COS1 cells were transfected with either a Lys-type plasmid or Asn-type plasmid, and the amounts of secreted ET-1 were measured by ELISA. Since ECE is expressed in many cell lines, including COS1 cells, preproET-1 should be converted to ET-1. In the culture supernatant collected from the cells transfected with either the Lys-type or Asn-type plasmid, higher levels of ET-1 were detected as compared with that in cells transfected with the vector alone (Fig. 2A, left panel). No difference in ET-1 level was observed between the Lys-type and Asn-type transfected cells (Fig 2A, left panel). We also measured the big ET-1 levels. The amount of big ET-1 in the supernatant was much higher than that of ET-1, and also was not significantly different between Asn-type and Lys-type transfected cells (Fig. 2A, right panel). Using co-transfected pMI-SEAP plasmid, we normalized the transfection efficiency of the plasmids, but the results were same.

Regarding the other cell lines, 293 cells and HUVECs, there were no significant differences in either the ET-1 or big ET-1 levels in the culture supernatant between Asn-type and Lys-type transfected cells, as indicated in COS1 cells (Fig. 2B, C). Normalization of the transfection efficiency by co-transfection of pMI-SEAP plasmid did not alter the results.

Relationship between *EDN1* Gene Polymorphism and Plasma ET-1 Level in Hypertensives

We assessed the difference in plasma ET-1 level between genotypes in Lys198Asn (5727G>T) and 3A/4A (−134delA) in 54 patients with essential hypertension (mean age: 61.0± 12.8 years old; male/female: 30/24; mean BMI: 25.3± 3.6 kg/m^2; mean BP: 144.5±17.3/82.2±10.5 mmHg). As shown in Fig. 3A, the plasma levels of ET-1 tended to be higher in subjects with the Asn-allele (GT+TT) than in homozygotes with 198Lys (GG), but the difference was not significant ($p=0.057$). In contrast, there was a significant difference in plasma ET-1 level between 3A/3A and 3A/4A (Fig. 3B). There were no differences in age, sex, BMI, BP levels or renal function between the two genotypes of these *EDN1* gene polymorphisms (data not shown).

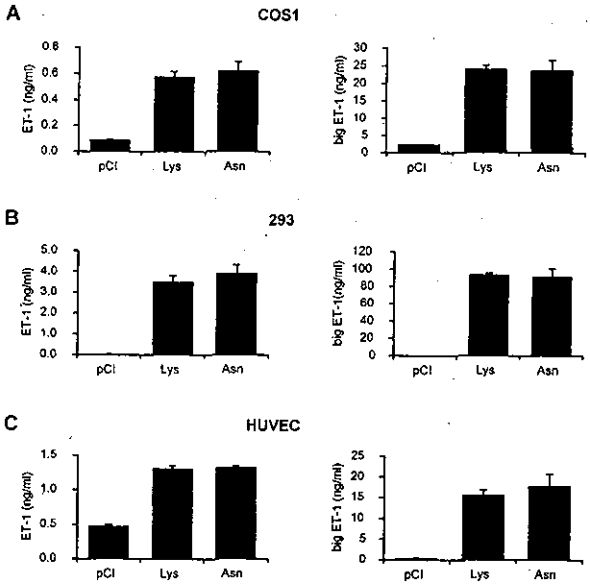

Fig. 2. *A: Levels of ET-1 (left panel) and big ET-1 (right panel) in the culture supernatant of COS1 cells. B: Levels of ET-1 (left panel) and big ET-1 (right panel) in the culture supernatant of 293 cells. C: Levels of ET-1 (left panel) and big ET-1 (right panel) in the culture supernatant of HUVECs. Each cell line was transfected with pCI/hET-1/Lys, pCI/hET-1/Asn or pCI. The levels of ET-1 and big ET-1 in the culture supernatant were measured by ELISA. The data represent the means±SD from three independent experiments. ET-1, endothelin-1; HUVECs, human umbilical vein endothelial cells.*

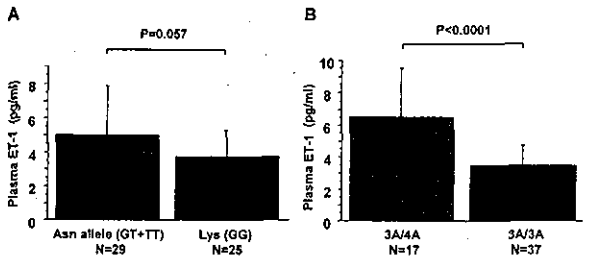

Fig. 3. *A: Comparison of plasma ET-1 level between Asn-allele and Lys homozygotes of the* EDN1 *Lys198Asn polymorphism (5727G>T). B: Comparison of plasma ET-1 level between 3A/3A and 3A/4A of the* EDN1 *3A/4A polymorphism in the 5′-untranslated region (−134delA). The data represent the means±SD. ET-1, endothelin-1.*

Discussion

Several reports have described that the Lys198Asn polymorphism in preproET-1 showed a positive association with BP elevation in overweight people, and this was observed not only in Caucasians but also in Japanese (*9–11*). Another study focusing on pregnant women showed that the Asn-allele was associated with SBP, and homozygotes with Asn198 had a significantly increased level of plasma ET-1 compared to women with other genotypes (*12*). These studies suggested that the Lys198Asn polymorphism may affect BP regulation through the production of ET-1. However, none of the studies examined the functional consequence of the Lys198Asn polymorphism on preproET-1. Because the Lys198Asn polymorphism is located in the coding region of the *EDN1* gene, it is likely that the polymorphism affects the processing of preproET-1 rather than modifying the gene expression or the stability of the mRNA. Here, we have designed a strategy to test this hypothesis by expressing the Lys-type and Asn-type preproET-1 transiently in three different cell lines, COS1 cells, 293 cells and HUVECs. The results showed that neither ET-1 nor big ET-1 levels in the culture supernatant of the Asn-type transfected cells were significantly changed compared to those of the Lys-type transfected cells.

The amount of big ET-1 in the medium was much higher than that of ET-1 in all cell types (Fig. 2). ET-1 is synthesized from big ET-1 through proteolytic cleavage by ECE. The large amount of big ET-1 in the culture supernatant indicated the inefficient cleavage of big ET-1 by endogenous ECE, because transient expression of preproET-1 using cytomegalovirus promoter would result in overproduction of big ET-1 for the endogenous enzyme.

It has been reported that ET-1[1–31], a peptide 10 amino acids longer than ET-1, is synthesized from big ET-1 by the proteolytic action of chymase and is also bioactive (*19–21*). The Lys198Asn polymorphism might affect the production of ET-1[1–31]. Unfortunately, in the present study, ET-1[1–31] could not be detected in the culture supernatant by ELISA (data not shown), probably due to the very low activity of chymase in the cells that we used.

In the measurement of the plasma ET-1 level in the 54 patients with essential hypertension, we were not able to find a significant difference between the genotypes in the *EDN1* Lys198Asn polymorphism, though the Asn-allele subjects tended to have a higher plasma ET-1 level than subjects with the Lys-allele. This finding seems to be concordant with the results obtained from the transfection study. Barden *et al.*, however, reported that plasma ET-1 levels were increased in Asn homozygotes in pregnant women (*12*). In addition, the positive correlation between this polymorphism and high BP has been observed only in overweight subjects (*9, 10*). Thus, it is suggested that a study focusing on normotensives is necessary, because the expression and/or production of ET-1 might be modified by BP or other factors such as obesity. Furthermore, the interaction between the *EDN1* Lys198Asn polymorphism and other factors, such as insulin resistance or activation of the renin-angiotensin system, may be involved in the mechanisms elevating BP *in vivo*.

An association between hypertension and the 3A/4A polymorphism (−134delA) located 138 bp downstream from the transcription start site in the 5′-untranslated region in the

EDN1 gene has been reported (*14*, *15*). Popowski *et al.* reported that the HUVECs obtained from homozygotes with the 4A-allele type showed a significantly increased expression of ET-1 protein due to enhanced mRNA stability (*16*). In the present study, significantly higher plasma ET-1 levels were observed in hypertensive subjects with the 4A-allele compared to 3A/3A homozygotes, although Popowski *et al.* could not find increased expression of ET-1 in the HUVECs obtained from heterozygotes. The reason why they did not observe the increased secretion of ET-1 in the *ex vivo* system was likely due to the difference in the background of the individuals—that is, they obtained their results using HUVECs from healthy German newborns and our results were obtained from Japanese hypertensives. LD analysis revealed that the Lys198Asn and 3A/4A polymorphisms are slightly in LD ($D'=0.65$, $r^2=0.20$). In the present study, the tendency for a positive correlation between the Lys198Asn polymorphism and plasma ET-1 levels was also recognized.

In conclusion, our results indicated that the *EDN1* Lys198Asn polymorphism did not directly affect ET-1 production in the expression assay using cultured transfected cells. Therefore, we propose that another variant having LD with this polymorphism, such as maybe the 3A/4A polymorphism, might be responsible for the association with BP elevation *via* plasma ET-1 up-regulation, or the interaction between the *EDN1* Lys198Asn polymorphism and other factors such as obesity might be involved in the mechanisms elevating BP *in vivo*.

Acknowledgements

We thank Dr. T. Sawamura at the Research Institute of the National Cardiovascular Center for providing human preproET-1 cDNA.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 29, Issue of July 16, pp. 30896–30903, 2004
Printed in U.S.A.

Identification of Strain-specific Variants of Mouse *Adamts13* Gene Encoding von Willebrand Factor-cleaving Protease*

Received for publication, December 26, 2003, and in revised form, May 7, 2004
Published, JBC Papers in Press, May 10, 2004, DOI 10.1074/jbc.M314184200

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Human *ADAMTS13* was recently identified as a gene encoding von Willebrand factor-cleaving protease, hADAMTS13. Both congenital and acquired defects in this enzyme can cause thrombotic thrombocytopenic purpura. hADAMTS13 consists of 1,427 amino acid residues and is composed of multiple structural domains including thrombospondin type 1 motifs and CUB domains. To analyze the functional roles of these domains *in vivo*, we determined the cDNA sequence of the mouse ortholog, mADAMTS13. Unexpectedly, two forms of the mouse *Adamts13* gene were isolated that differed in the insertion of an intracisternal A particle (IAP) retrotransposon including a premature stop codon. The IAP insertion was found in BALB/c, C3H/He, C57BL/6, and DBA/2 strains but not in the 129/Sv strain. The outbred ICR strain had either the IAP-free or IAP-inserted allele or both. IAP-free *Adamts13* encoded mADAMTS13L, a protein of 1,426 amino acid residues with the same domain organization as hADAMTS13. In contrast, IAP-inserted *Adamts13* encoded a C-terminally truncated enzyme, mADAMTS13S, that is comprised of only 1,037 amino acid residues and lacking the C-terminal two thrombospondin type 1 motifs and two CUB domains. Strain specificity was also confirmed by reverse transcription-PCR and Northern blot analyses. Both recombinant mADAMTS13L and mADAMTS13S exhibited von Willebrand factor cleaving activities *in vitro*. The natural variation in mouse ADAMTS13 should allow for the determination of hitherto unknown functions of its C-terminal domains *in vivo*.

von Willebrand factor (VWF)[1] is a large glycoprotein that mediates adhesion between the platelet surface and damaged subendothelium (1, 2). VWF is mainly synthesized in endothelial cells and secreted into the circulating blood as unusually large VWF (UL-VWF) multimers (1, 2). In healthy individuals, UL-VWF multimers are cleaved to smaller sizes in plasma (3). If cleavage is impaired, however, UL-VWF multimers accumulate in the plasma. Because UL-VWF multimers possess an extremely high thrombotic activity (4, 5), UL-VWF multimers in the circulation lead to platelet clumping at the sites of vascular injury. The importance of VWF proteolysis is best illustrated by the severe consequences of thrombotic thrombocytopenic purpura, a condition associated with increased levels of UL-VWF multimers (6). This disease is characterized by microangiopathic hemolytic anemia, thrombocytopenia, neurological dysfunction, renal failure, and fever (7). The mortality of affected patients may exceed 90% without treatment such as plasma exchange.

Human ADAMTS13 (hADAMTS13), an enzyme responsible for the proteolytic processing of UL-VWF multimers, was recently purified, and its partial amino acid sequence was determined (8–10). hADAMTS13 cleaves a peptidyl bond between Tyr^{1605} and Met^{1606} in the VWF A2 domain (11–13). The gene encoding hADAMTS13 was identified as a member of the "a disintegrin-like and metalloprotease with thrombospondin type 1 motif (ADAMTS)" family and designated as *ADAMTS13* (8, 14, 15). *ADAMTS13* contains 29 exons and spans ~37 kb on chromosome 9q34 (8, 14, 15). The mRNA is detected primarily in liver (8, 14, 15). Analysis of genomic DNA in patients with congenital thrombotic thrombocytopenic purpura revealed that mutations of *ADAMTS13* could lead to an inactive enzyme (15–20). Notably, a common single nucleotide polymorphism, P475S, with ~10% heterozygosity in the Japanese population, resulted in a decrease of enzymatic activity (16).

hADAMTS13 consists of several different domains: a signal peptide, a propeptide, a reprolysin-like metalloprotease domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) motif, a cysteine-rich domain, a spacer domain, seven additional TSP1 repeats, and two CUB domains. *In vitro* studies using C-terminally truncated hADAMTS13 constructs revealed that the C-terminal TSP1 motifs and CUB domains were dispensable to maintain the VWF cleaving activity (21, 22). However, the biochemical and physiological roles of these domains *in vivo* remain to be resolved. As a first step to develop suitable animal models for following potential roles of this enzyme *in vivo*, we have cloned the mouse ortholog of hADAMTS13, mADAMTS13, and determined its complete genomic structure. In the present study, we report two types of the *Adamts13* gene in mice caused by the strain-specific insertion of an intracisternal A-particle (IAP) retrotransposon. We further examine the VWF cleaving activity of mADAMTS13.

* This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; from the Japan Society for the Promotion of Science; and from the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB071302, AB095445, and AB112362.

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[1] The abbreviations used are: VWF, von Willebrand factor; TSP1, thrombospondin type 1; UL-VWF, unusually large VWF; IAP, intracisternal A-particle; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; ORF, open reading frame; h, human; m, mouse; HRP, horseradish peroxidase.

EXPERIMENTAL PROCEDURES

Animals—Male 129/Sv mice were purchased from Clea Japan, Inc. Male BALB/c, C3H/He, C57BL/6, DBA/2, and ICR mice were purchased from Japan SLC, Inc. Blood (~100 μl) was collected by cardiac puncture into a syringe containing 10 μl of 3.8% sodium citrate and centrifuged to obtain plasma. Spleen and liver were excised, rinsed in phosphate-buffered saline, and immediately used for DNA and RNA preparation.

DNA Sequencing—All of the sequence analyses were performed by 373A or 3700 automated DNA sequencer (Applied Biosystems) with a BigDye Terminator Kit (Applied Biosystems).

Determination of the mADAMTS13 cDNA Sequence—Total RNA was prepared from the livers of C57BL/6 and 129/Sv mice with Isogen (Nippon Gene), and poly(A)$^+$ RNA was purified with an mRNA purification kit (Amersham Biosciences) according to the manufacturer's instruction. The cDNA was synthesized from the poly(A)$^+$ RNA with a first strand cDNA synthesis kit (Amersham Biosciences). PCR was carried out with primers designed from the genomic DNA sequence (forward sequence in 5′-untranslated region, 5′-AGGAAGCTCCCAAGAGTAAACACTGCCT-3′; reverse sequence within the metalloprotease domain, 5′-TCAGAGAGGGTGATTAGCTTCACCAGGT-3′). PCR products were cloned into pCR2.1 vector using a TA Cloning™ kit (Invitrogen) and sequenced.

In addition, 3′-RACE was performed using a 3′-Full RACE Core Set (Takara), according to the manufacturer's instructions. After reverse transcription from liver poly(A)$^+$ RNA, PCR was performed using the Adaptor Primer provided with the kit and a gene-specific forward primer within the metalloprotease domain, 5′-TGGAGTTGCCTGATGGCAACCAGCA-3′. The second PCR was performed using the first PCR products as a template with Adaptor Primer and a gene-specific internal forward primer, 5′-CATCACCTTTTCCTACTTTCAACTGAAGCAG-3′. The cycling parameters were as follows: 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, followed by 72 °C for 7 min. PCR products were cloned into pCR2.1 vector and sequenced.

PCR Analysis of IAP Insertion in the Adamts13 Gene—Genomic DNA was extracted from ear punches of 129/Sv, BALB/c, C3H/He, C57BL/6, DBA/2, and ICR mice by DNeasy Tissue Kit (Qiagen). Presence or absence of an IAP insertion in the *Adamts13* gene was determined by PCR with HotStarTaq DNA polymerase (Qiagen). The amplification was carried out using mixture of three primers; the intron 23-specific forward primer, 5′-ACCTCTCAAGTGTTTGGGATGCTA-3′, the IAP-specific reverse primer, 5′-TCAGCGCCATCTTGTGACGGCGAA-3′, and the primer downstream of the IAP target site, 5′-TGCCAGATGGCCATGATTAACTCT-3′. PCR products were directly sequenced.

Southern Blot Analysis—Genomic DNA prepared from spleens was digested with EcoRV, separated on 0.7% agarose gel, and transferred to a nylon membrane by standard capillary blotting techniques. A genomic fragment (842 bp) upstream of the IAP target site was produced by PCR with the primers 5′-TAGGCAGCCATGGATCTGTATTAG-3′ and 5′-TGTCTGCTCTTCCAGAAATCCTTA-3′ and labeled with fluorescein-11-dUTP using Gene Images random prime labeling module (Amersham Biosciences). The blot was hybridized with the probe, and the hybridized probe was detected using the Antifluorescein-AP conjugate and the CDP-*Star* detection reagent (Amersham Biosciences) according to the manufacturer's instructions. Chemiluminescence was measured by an LAS-1000plus image analyzer (Fujifilm).

Determination of the Adamts13 Genomic Sequence—A λ phage library constructed from Sau3AI-digested genomic DNA of the 129/Sv strain was screened by a PCR-based method as described previously (23). Three independent positive phages were obtained. Each phage insert DNA was subcloned into pBluescript *II SK(+)* vector (Stratagene) and sequenced using the GPS-1 Genome Priming System (New England Biolabs) according to the manufacturer's instructions. The sequence data were assembled and analyzed using the Sequencher software (Gene Codes). Sequence gaps of each target DNA were filled by primer walking sequencing.

Reverse Transcription-PCR—Total RNA was extracted from the livers of six mouse strains with Isogen (Nippon Gene), and poly(A)$^+$ RNA was purified using PolyATtract mRNA Isolation Systems (Promega) and subjected to one-step RT-PCR (Qiagen). The exon 21/22-specific sense primer (5′-TTGTGGGAGAGGTCTGAAGGAACT-3′), the pseudo-exon 24-specific antisense primer (5′-TCAGCGCCATCTTGTGACGGCGAA-3′), and the exon 24/25-specific antisense primer (5′-ACAGGAGACAGAGCACTCTGTCCA-3′) were simultaneously used for the amplification. The PCR products were excised from the agarose gel and sequenced.

Northern Blot Analysis—The specific fluorescein-labeled probe (1.3 kb) was synthesized by PCR from mouse *Adamts13* cDNA as described previously (23). The primers used were a sense primer located in exon 3 (5′-ATTCTGCACCTGGAACTCCTGGTA-3′) and an antisense primer located in exon 13 (5′-CGGCTGACAATGAAGCTTTCTCCA-3′). Poly(A)$^+$ RNA (10 μg) from mouse livers were separated on 1% agarose gel containing 2% formaldehyde and transferred to a nylon membrane. Hybridization and detection using the Antifluorescein-AP conjugate and the CDP-*Star* detection reagent (Amersham Biosciences) were performed according to the manufacturer's instructions. Commercially available premade Northern blot membranes containing poly(A)$^+$ RNA from the BALB/c strain (Multiple Tissue Northern blot; Clontech) and the Swiss Webster strain (FirstChoice Northern blot; Ambion) were also analyzed using the Antifluorescein-HRP conjugate and the DNA Thunder Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Chemiluminescence was measured by an LAS-1000plus image analyzer (Fujifilm).

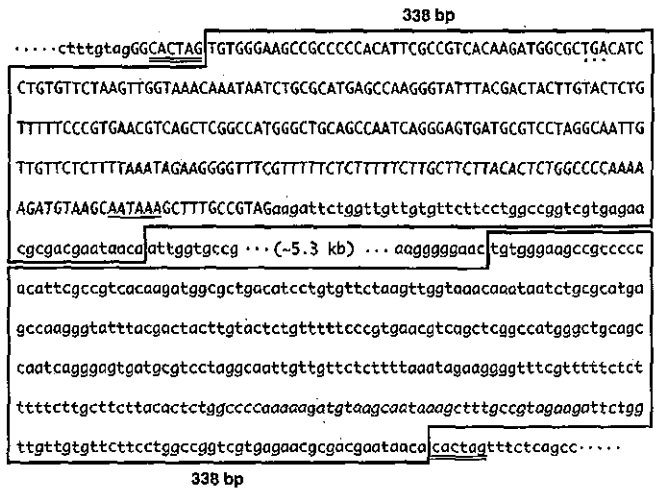


FIG. 1. **Nucleotide sequence of the retrovirus-like element observed in *Adamts13* intron 23 of the C57BL/6 strain.** The 338-bp repetitive sequences of a retrovirus-like element are *boxed*. The 6-bp duplication of target DNA at the insertion site is *double underlined*. Exon 24 of the C57BL/6 strain, shown in *uppercase letters*, contains a stop codon TGA (*dotted*) and a putative polyadenylation signal AATAAA (*underlined*).

Preparation of Recombinant Substrate (mVWF73) for Enzymatic Assay—To examine the enzymatic activities of mADAMTS13, we prepared the recombinant substrate as described previously (24). In brief, a D1596-T1668 region of mouse VWF was amplified by RT-PCR using total RNA from a C57BL/6 mouse liver. The primers, 5′-cgggatccGACCGGGTAGAGGCACCTAACC-3′ and 5′-cggaattcTCAGTGATGGTGATGGTGATGTGTCTGCAGGACCAGGTCAGGA-3′ were used for the amplification. Lowercase letters indicate added restriction enzyme sites, and the underlined sequence is the inserted C-terminal His_6 tag (H). The PCR product was digested with BamHI and EcoRI and cloned into the corresponding sites of pGEX-6P-1 (Amersham Biosciences), a glutathione S-transferase (GST) fusion expression vector. The resulting plasmid encoding GST-D1596T1668-H was introduced into *Escherichia coli*, BL21 (Stratagene), and expression was induced by the addition of isopropyl-β-D-thiogalactoside. The bacterial cells were collected and lysed with CelLytic B (Sigma), followed by centrifugation. The soluble fraction was subjected to a nickel-nitrilotriacetic acid Spin Kit (Qiagen) and further to a MicroSpin GST purification module (Amersham Biosciences). The purified protein, designated GST-mVWF73-H, was used as substrates for enzymatic assays. The molecular mass of GST-mVWF73-H was 35.7 kDa. If mADAMTS13 cleaves the expected site, the size of the N-terminal portion including the GST tag will be 28.0 kDa.

Transient Expression of mADAMTS13—The entire open reading frame (ORF) constructs with C-terminal FLAG sequence (DYKDDDDK) were prepared for two types of mouse ADAMTS13, mADAMTS13L (GenBank™ accession number AB112362) and mADAMTS13S (GenBank™ accession number AB071302), by PCR. Each PCR product was inserted into pCAGG-neo mammalian expression vector (25). The resulting plasmids were transfected into HeLa cells using FuGENE 6 (Roche Applied Science) as described previously (16). Forty-eight hours after transfection, the media were collected and concentrated using Centricon YM-30 (Millipore). The cells together with extracellular matrix were lysed in SDS sample buffer (10 mM

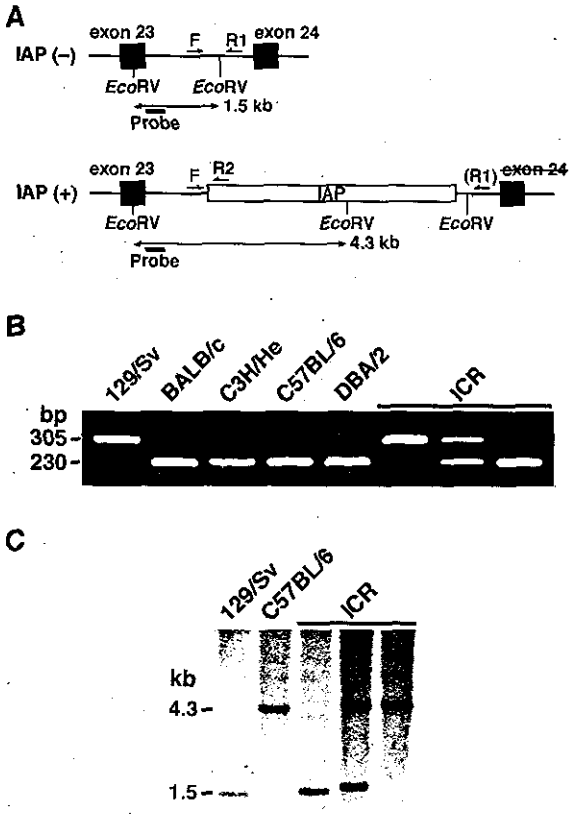
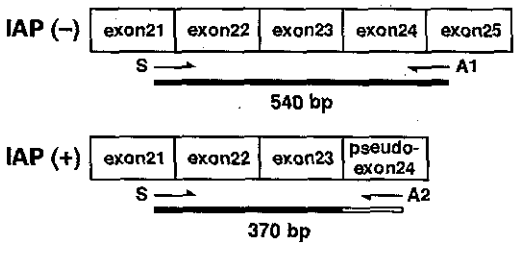


FIG. 2. **Genotyping of mouse *Adamts13*.** *A*, diagram of a segment of the *Adamts13* gene around the IAP insertion site. The sites of primers used for the genotyping PCR are indicated by *arrows*. The EcoRV fragments detected in Southern blot analysis are indicated by *double-headed arrows*. *B*, PCR analysis. In mixture of three primers, F and R1 primers generate a 305-bp product specific for the IAP-free *Adamts13* gene, whereas F and R2 primers generate a 230-bp product specific for the IAP-inserted *Adamts13* gene. *C*, Southern blot analysis. Genomic DNA from each mouse strain was digested with EcoRV and hybridized with the probe that detects a 1.5-kb fragment in the IAP-free allele and a 4.3-kb fragment in the IAP-inserted allele.



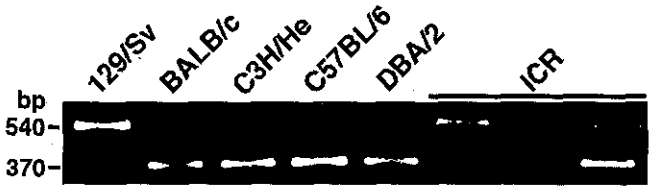


FIG. 3. **RT-PCR of *Adamts13* mRNA in liver.** PCR primers are shown as *arrows* indicating direction at their approximate locations. In combination of three primers, S and A1 primers generate a 540-bp product specific to the IAP-free transcript, whereas primers S and A2 generate a 370-bp product specific to the IAP-inserted transcript.

Tris-HCl, 2% SDS, 50 mM dithiothreitol, 2 mM EDTA, 0.02% bromphenol blue, 6% glycerol, pH 6.8).

Recombinant proteins were detected by SDS-PAGE and Western blot as described previously (16). For culture media, a rabbit anti-

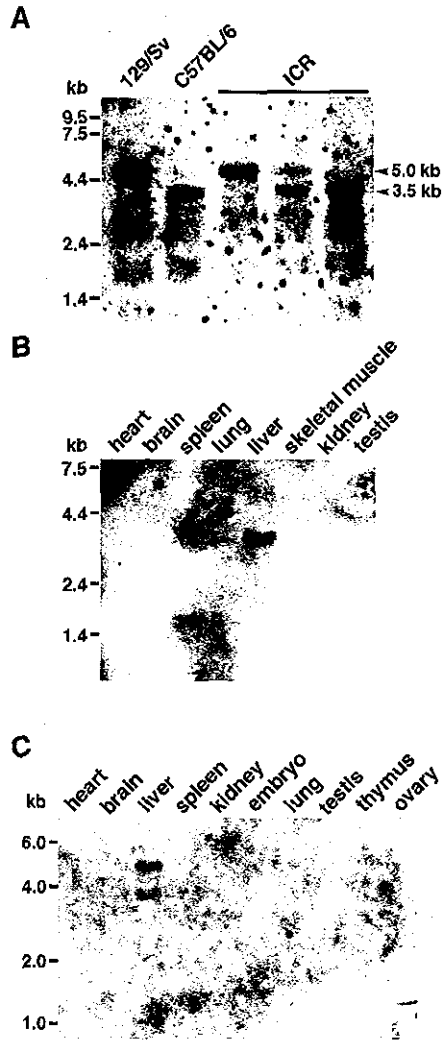


FIG. 4. **Northern blot analysis of mouse *Adamts13* mRNA.** *A*, expression of *Adamts13* mRNA in liver. Poly(A)$^+$ RNA isolated from liver of indicated strains was probed with a 1.3-kb *Adamts13* cDNA corresponding to exons 3–13. The approximate sizes of the IAP-free (5.0 kb) and the IAP chimeric (3.5 kb) transcripts are indicated by *arrowheads*. *B*, expression of *Adamts13* mRNA in tissues from BALB/c mice. *C*, expression of *Adamts13* mRNA in tissues from SwissWebster mice. The sizes of RNA markers are shown at the *left*.

mADAMTS13 polyclonal antibody (described below) and an HRP-labeled goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories) were used for detection after SDS-PAGE under nonreducing condition. For cell lysates, an anti-FLAG M2 monoclonal antibody (Sigma) and an HRP-labeled goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories) were used for detection after SDS-PAGE under reducing condition. Chemiluminescence was developed using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and detected by an LAS-1000plus image analyzer (Fujifilm).

Preparation of Polyclonal Antibody against Mouse ADAMTS13—A polyclonal antiserum against mADAMTS13 was raised by DNA-based immunization protocols. Rabbits were immunized by intradermal injection with ~1 mg of mADAMTS13S expression plasmid at 25 sites on the back. Booster immunizations were carried out by the same protocol 3 weeks after the primary immunization. Serum was collected 3 weeks after the second immunization. The IgG fraction was then prepared by an affinity chromatography using a protein G column (Amersham Biosciences).

Enzymatic Assay—Purified GST-mVWF73-H (500 ng) was incubated with recombinant mADAMTS13L or mADAMTS13S in 40 μl of reaction buffer (5 mM Tris-HCl, 10 mM $BaCl_2$, 0.01% Tween 20, and 1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, pH 8.0) at 37 °C for 1 h. The reaction was stopped by adding 10 μl of SDS sample buffer (50 mM Tris-HCl, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA, 0.1% bromphenol blue, 30% glycerol, pH 6.8). The samples were sub-

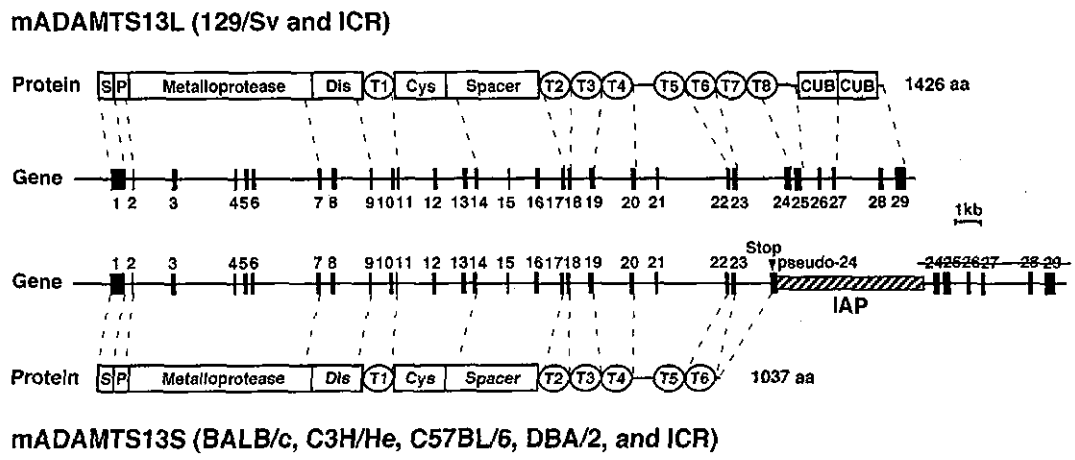


FIG. 5. **Schematic structure of two forms of the *Adamts13* genes and proteins.** Genomic and protein structures of mADAMTS13L in the 129/Sv strain and of mADAMTS13S in the BALB/c, C3H/He, C57BL/6, and DBA/2 strains are shown. The outbred ICR strain has both type alleles. The exons and introns are drawn to scale. A *hatched box* represents the IAP insertion in intron 23. *S*, signal peptide; *P*, propeptide; *Dis*, disintegrin-like domain; *T1–T8*, thrombospondin type1 motifs; *Cys*, cysteine-rich domain.

jected to Western blot using a rabbit anti-GST antibody (Molecular Probes) and an HRP-labeled goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories) as described (24). We also analyzed the proteolytic activity of plasma from five mouse strains in the same way.

RESULTS

Identification of Adamts13 in the C57BL/6 Strain—To identify the orthologous mouse gene of human *ADAMTS13*, we performed a BLAST search in the public data base, based on the human *ADAMTS13* cDNA sequence (GenBank™ accession number AB069698, 4,284-nucleotide ORF) reported by Soejima *et al.* (8). This search led us to identify a compatible genomic sequence (GenBank™ accession number AC090008) derived from the C57BL/6 strain. This sequence was located on chromosome 2, band A3 and contained 29 conserved exons similar to human *ADAMTS13*. To obtain the cDNA to corresponding mRNA, we performed RT-PCR and 3′-RACE using poly(A)$^+$ RNA from the liver of a C57BL/6 mouse. Unexpectedly, the cDNA sequence (GenBank™ accession number AB071302) included only a 3,114-nucleotide ORF derived from 24 exons.

From a comparison between the cDNA and the genomic sequences of the C57BL/6 strain, we found a 6-kb retrovirus-like sequence in intron 23 of the *Adamts13* gene (Fig. 1). This sequence was flanked by the identical 338-bp sequence with a 6-bp (CACTAG) duplication of target site, as is often observed for retrotransposition. A BLAST search identified the insertional element as an intracisternal A-particle (IAP), which is one of the retrotransposons present at about 2,000 sites in the mouse genome (26). This insertion of IAP seemed to be responsible for loss of the original mRNA 3′-end by splicing exon 23 to pseudo-exon 24 that contains a premature stop codon.

Identification of Adamts13 in the 129/Sv Strain—To determine whether the IAP insertion into the *Adamts13* gene is common to a variety of mouse strains, we carried out PCR genotyping of five inbred and one outbred strains (Fig. 2*A*). A mixture of three primers was used for the reaction; Primers F and R1 were designed to produce a 305-bp band in the absence of IAP, and primers F and R2 were designed to produce a 230-bp band in the presence of IAP. This experiment revealed that the IAP insertion was present in the BALB/c, C3H/He, and DBA/2 strains but not in the129/Sv strain (Fig. 2*B*). The outbred ICR strain was genetically heterogeneous with respect to the IAP insertion into the *Adamts13* gene. Southern blot analysis using probes upstream of the IAP target site revealed that *Adamts13* was a single copy gene and confirmed the strain-specific insertion of IAP (Fig. 2*C*). These results implied that the *Adamts13* gene transcript of the 129/Sv mice and some ICR mice might contain residual exons lost in the other mouse strains.

To confirm this hypothesis, RT-PCR was performed using liver poly(A)$^+$ RNA from a 129/Sv mouse. The obtained sequences indicated that the *Adamts13* cDNA (GenBank™ accession number AB112362) contained a 4,281-nucleotide ORF similar to human *ADAMTS13* (4,284-nucleotide ORF). To determine the complete genomic sequence of *Adamts13* in the 129/Sv strain, we screened a 129/Sv mouse λ genomic library. Sequence analysis of positive phage clones confirmed the absence of IAP in the *Adamts13* gene. *Adamts13* in the 129/Sv strain (GenBank™ accession number AB095445) contained 29 exons like human *ADAMTS13* and spanned ~30 kb.

To examine the effect of IAP on *Adamts13* mRNA splicing, RT-PCR was performed using liver poly(A)$^+$ RNA from six mouse strains (Fig. 3). The exon 21/22-specific sense primer, the exon 24/25-specific antisense primer, and the pseudo-exon 24-specific antisense primer were mixed and used for the amplification. We detected the IAP chimeric transcript in four inbred strains with the IAP insertion. In contrast, the IAP-free transcript was observed in the 129/Sv strain. The heterogeneous expression of two types of transcripts was observed in samples from the ICR strain. To characterize the transcripts in more detail, Northern blot analysis of liver RNA was carried out using a 1.3-kb probe spanning exons 3–13 of *Adamts13* cDNA (Fig. 4*A*). The RNA was prepared from the same animals as used for the Southern blot analysis. An ~3.5-kb mRNA corresponding to the size of IAP chimeric transcript was detected in the C57BL/6 and the ICR strains. The IAP-free transcript of ~5.0 kb was observed in the 129/Sv and the ICR strains.

Thus, these results clearly indicate the presence of two types of mouse *Adamts13* in a strain-specific manner (Fig. 5). *Adamts13* of the 129/Sv strain encodes an ADAMTS13 protein containing 1,426 amino acid residues with the same domain structure as hADAMTS13, designated mADAMTS13L. *Adamts13* of the BALB/c, C3H/He, C57BL/6, and DBA/2 strains encodes the shorter ADAMTS13 protein including only 1,037 amino acid residues, designated mADAMTS13S. In this protein, the C-terminal two TSP1 and two CUB domains are replaced with the 16-amino acid sequence, ALVWEAAPTFAVTRWR, derived from the IAP. The outbred ICR strain carries either the IAP-free or IAP-inserted allele or both.

Expression of the Adamts13 mRNA in Mouse Tissues—To study the expression pattern of the mouse *Adamts13* gene, we analyzed Northern blots containing poly(A)$^+$ RNA from various tissues of the BALB/c and the Swiss Webster strains. As shown

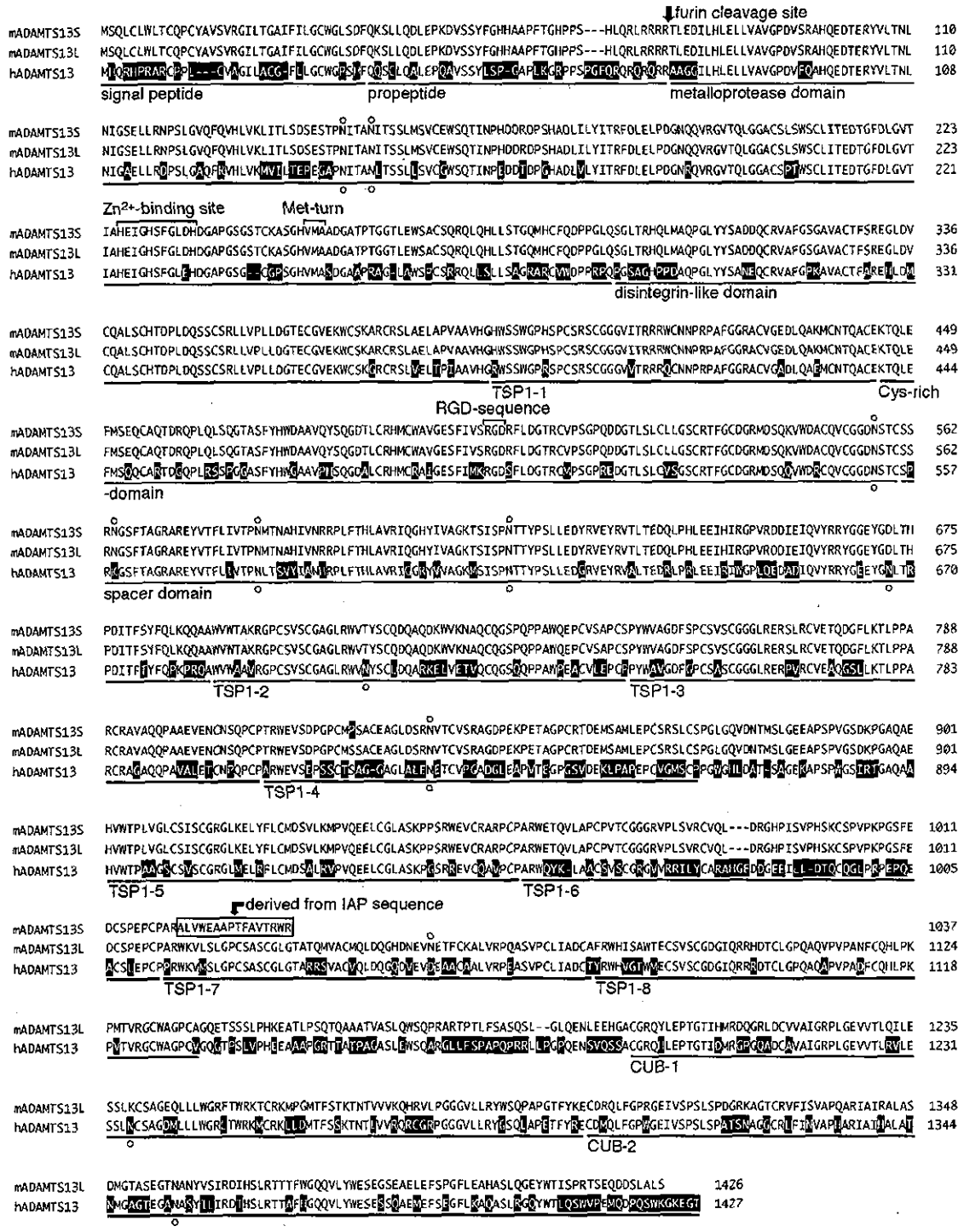


FIG. 6. **Alignment of deduced amino acid sequences of mADAMTS13 and hADAMTS13.** Sequences are represented with the *single-letter code*, and residues that differ from mADAMTS13L are *shaded*. Each structural domain is *underlined*. The predicted furin cleavage site (R*X*(K/R)R) is marked with an *arrow*. The IAP-derived 16 amino acid residues in mADAMTS13S are *boxed*. *Open circles* indicate the potential *N*-glycosylation sites.

in Fig. 4 (*B* and *C*), *Adamts13* mRNA in both strains was exclusively observed in the liver, suggesting that mADAMTS13 is primarily synthesized in the liver, similar to hADAMTS13. A single transcript of 3.5 kb was expressed in liver of the BALB/c strain with the IAP insertion. In contrast, two transcripts of 5.0 and 3.5 kb were detected in liver of the outbred Swiss Webster strain, suggesting that this strain may carry two types of alleles, like the ICR strain. Both SwissWebster and ICR strains are derived from a colony of Swiss mice.

Comparison of the Deduced Amino Acid Sequences of mADAMTS13 and hADAMTS13—The deduced amino acid sequences of mouse and human ADAMTS13 were aligned (Fig. 6). The overall sequence identity between mADAMTS13L and hADAMTS13 was ~70%. The highest identity (>80%) was observed in the disintegrin-like (81%), TSP1–1 (89%), cysteine-rich (80%), and TSP1–8 (83%) domains, whereas relatively low conservation (<60%) was observed in the signal peptide (43%), propeptide (52%), TSP1–4 (46%), and TSP1–6 (40%) domains. mADAMTS13L contained eight potential *N*-glycosylation sites, six of which were conserved in hADAMTS13. mADAMTS13L also included several motifs characteristic for each domain of hADAMTS13, such as a furin cleavage sequence at the end of

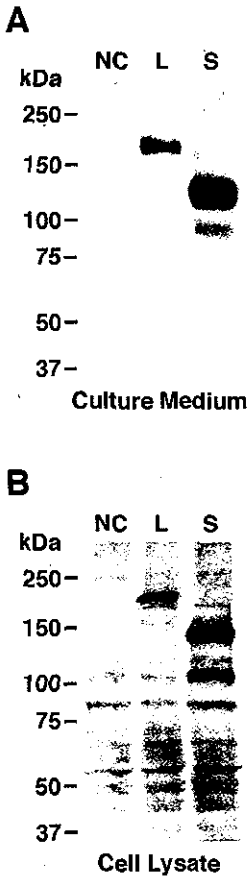


FIG. 7. **Transient expression of recombinant mADAMTS13.** *A*, mADAMTS13 in the culture medium. HeLa cells were transfected with plasmids encoding mADAMTS13L (*L*) and mADAMTS13S (*S*). The concentrated culture media were analyzed by Western blot with an anti-mADAMTS13 antibody under nonreducing conditions. *B*, mADAMTS13 in the cell lysate. The cell lysates including extracellular matrixes were analyzed by Western blot with an anti-FLAG antibody under reducing condition. *NC* is the culture medium or the lysate of untransfected cells. The size of protein markers is indicated at the *left*. The small size difference of recombinant enzymes in medium and cell lysates was due to the difference of electrophoretic condition. The faster migrating bands seen in medium and cell lysates expressing mADAMTS13S may result from proteolysis during cell culture. The typical result of three experiments is shown.

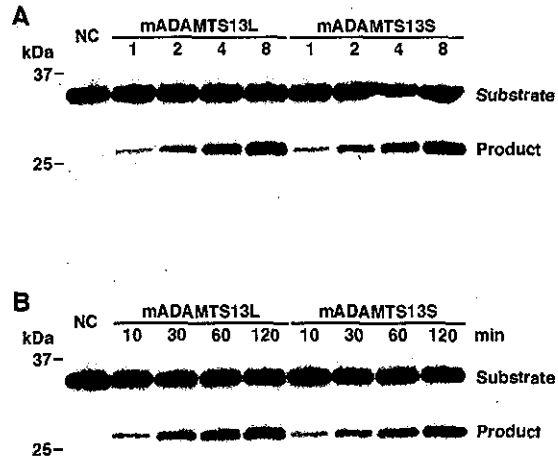


FIG. 8. **Enzymatic activity of recombinant mADAMTS13.** *A*, *cleavage of* GST-*m*VWF73-H by serial dilutions of mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S at 37 °C for 1 h. A negative control reaction using the culture medium of untransfected cells (NC) was also performed simultaneously. The products were analyzed by Western blot using an anti-GST antibody. The *numbers 1, 2, 4,* and *8* indicate relative amounts of mADAMTS13L and mADAMTS13S in the reaction mixtures. The typical result of three experiments is shown. *B*, time course of GST-mVWF73-H cleavage by mADAMTS13. GST-mVWF73-H was incubated with recombinant mADAMTS13L or mADAMTS13S for the indicated time at 37 °C. The reaction mixtures contained the equivalent amounts of the recombinant enzyme. Products were analyzed by Western blot using an anti-GST antibody. The typical result of three experiments is shown.

the propeptide, a zinc-binding site in the metalloprotease domain, and an RGD sequence in the cysteine-rich domain. The sequences of mADAMTS13L and mADAMTS13S were almost identical, except for the deletion of C-terminal regions.

Expression and Enzymatic Activity of Recombinant mADAMTS13L and mADAMTS13S—We transiently expressed the mADAMTS13L and mADAMTS13S proteins in HeLa cells. Western blot analysis using a polyclonal antibody against mADAMTS13 revealed that both were secreted into the culture media (Fig. 7*A*). Transient expression of mADAMTS13L produced an immunoreactive band of ~200 kDa, and mADAMTS13S exhibited a 130-kDa band. The level of mADAMTS13S in the medium was almost 10-fold higher than that of mADAMTS13L. This was not due to a preferential accumulation of mADAMTS13L on the cell surface or the extracellular matrix, because a relatively high amount of mADAMTS13S was also observed in the cell lysates (Fig. 7*B*). It was conceivable that mADAMTS13S was effectively synthesized in HeLa cells compared with mADAMTS13L in our experimental conditions. Whether mADAMTS13S is also preferentially expressed *in vivo* remains unknown. Further analysis is required to determine the plasma levels of mADAMTS13L and mADAMTS13S in mice.

The VWF cleaving activities of recombinant proteins were measured by the degradation of the specific recombinant substrate, GST-*m*VWF73-H. The relative concentration of recombinant mADAMTS13 in the culture medium was determined by chemiluminescent intensities on Western blot, and equal amounts were used for the enzymatic assay. The substrate, GST-mVWF73-H, was incubated with serial dilutions of the culture medium, and the cleavage product including the N-terminal GST tag was visualized by Western blot using anti-GST (Fig. 8). When the substrate was incubated with the medium of mADAMTS13L-transfected cells, a band appeared with the expected size of the N-terminal portion (28 kDa) in a concentration-dependent manner, indicating the cleaving activity of recombinant mADAMTS13L (Fig. 8*A*). No degradation was observed after the incubation of GST-mVWF73-H with the medium from untransfected cells. The cleaved band was also detected after incubation with the mADAMTS13S culture medium, and the chemiluminescent intensities of the product bands were almost equal to those obtained by mADAMTS13L (Fig. 8*A*). We confirmed that the degradation of GST-mVWF73-H by mADAMTS13 was also time-dependent, and the rate of the product formation by mADAMTS13S was similar to that by mADAMTS13L (Fig. 8*B*).

The VWF Cleaving Activity of Mouse Plasma—To examine the ADAMTS13 activity in plasma from various mouse strains, we collected plasma samples from five strains and carried out the enzymatic assay using GST-mVWF73-H. As shown in Fig. 9, plasma from all strains cleaved GST-mVWF73-H. Comparison of the product levels did not reveal a significant difference among strains. This suggested that the IAP insertion into the *Adamts13* gene does not affect the *in vitro* cleavage of GST-mVWF73-H by plasma.

DISCUSSION

In this study, we identified two isoforms of the mouse *Adamts13* gene that result from the strain-specific insertion of

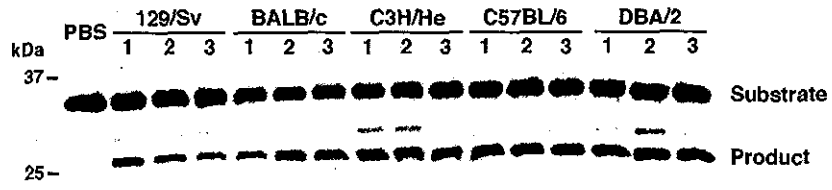


FIG. 9. **Cleavage of GST-mVWF73-H by mouse plasma.** GST-mVWF73-H was incubated with plasma samples from mice with (BALB/c, C3H/He, C57BL/6, and DBA/2) or without (129/Sv) the IAP insertion in the *Adamts13* gene. The products were analyzed by Western blot using an anti-GST antibody. The results from three animals/strain are shown.

an IAP-retrotransposon. The IAP-free *Adamts13* gene contained 29 exons, and the deduced protein sequence included 1,426 amino acid residues with the same domain organization as hADAMTS13. In contrast, the IAP-inserted *Adamts13* gene contained only 24 exons encoding 1,037 amino acids having a truncated C terminus.

The inserted IAP is one of the endogenous transposable elements, which is closely related to retroviruses and transposed via the reverse transcription of an RNA intermediate (26, 27). The IAP element contains two long terminal repeats with the signals for the initiation/regulation of transcription and for the polyadenylation of transcripts (28). IAP insertions into introns have been shown to cause formation of chimeric transcripts (29–32), similar to our findings in the *Adamts13* gene. We noted that the presence of IAP in the *Adamts13* gene induces the appearance of a cryptic splicing site followed by a premature in-frame stop codon and a polyadenylation signal derived from the IAP long terminal repeat. As a result, the insertion leads to replacement of the last 405 amino acid residues corresponding to two TSP1 motifs and two CUB domains with the IAP-encoded 16 amino acid residues.

Northern blot and RT-PCR analyses confirmed that the IAP chimeric short transcript (3.5 kb) and the IAP-free long transcript (5 kb) were expressed in a strain-specific manner. Both types of transcripts were specifically expressed in the liver, consistent with expression of the human *ADAMTS13* gene. It should be noted that the IAP insertion could not completely abolish the formation of mADAMTS13L mRNA. The RT-PCR products (540-bp; Fig. 3) characteristic of mADAMTS13L mRNA were also detectable in the strains with the IAP insertion when using a large amount of template (data not shown). A small amount of mADAMTS13L protein may be expressed in mice with the IAP-inserted *Adamts13* gene such as the BALB/c, C3H/He, C57BL/6, and DBA/2 strains. Incidentally, the RT-PCR and 3′-RACE data did not show any splicing variants that encoded mADAMTS13S-like protein in the IAP-free strains.

Recently, we developed a novel recombinant substrate, GST-VWF73-H, to measure hADAMTS13 activity (24). GST-VWF73-H is a partial region of human VWF flanked by GST and His_6 tags. Because of difficulty in isolating VWF from mouse plasma, we have also prepared the recombinant substrate, GST-mVWF73-H, based on the mouse VWF cDNA sequence. Both mouse and human plasma efficiently cleaved GST-mVWF73-H and produced a fragment of the expected size. Mouse plasma also cleaved the substrate for hADAMTS13, GST-VWF73-H (data not shown).

Both recombinant mADAMTS13L and mADAMTS13S were secreted into the culture medium of HeLa cells. This result indicates that the IAP insertion does not abolish secretion of mADAMTS13 from cells. The recombinant mADAMTS13L and mADAMTS13S cleaved GST-mVWF73-H with nearly the same efficiency. Similarly, a deletion mutant of hADAMTS13 in mimicry of mADAMTS13S was also secreted efficiently from HeLa cells and cleaved GST-VWF73-H with normal activity (data not shown). In previous reports, we and others found that deletion mutants of hADAMTS13 devoid of the C-terminal TSP1 motifs and CUB domains retained VWF cleaving activity (21, 22). Therefore, our current observation on mouse and human recombinant proteins was consistent with these previous studies. Moreover, the plasma VWF cleaving activities in mice were also comparable among the strains with or without the IAP insertion in the *Adamts13* gene. The C-terminal two TSP1 motifs and two CUB domains of mADAMTS13 may contribute to activity but are not essential for the VWF cleavage, at least *in vitro*.

The fact that several common strains of mice have a naturally truncated form of ADAMTS13 allows us to hypothesize that the truncated domains are not necessary *in vivo*. However, several mutations in TSP1–7, TSP1–8, CUB-1, and CUB-2 domains of hADAMTS13 were reported to associate with congenital thrombotic thrombocytopenic purpura (15, 17–20). It is still unclear whether these mutants are secreted from cells, as is the case with mADAMTS13S. To date, two mutations, R1123C and 4143insA, were characterized by expression analysis, and both impaired secretion of the enzyme from cells (19, 20). The C-terminal mutations found in thrombotic thrombocytopenic purpura patients may influence their synthesis or secretion.

Bernardo *et al.* (33) reported that several short peptides within the regions from TSP1–6 to the C terminus of hADAMTS13 block VWF cleavage on the endothelial cell surface under flow conditions. This finding suggests an important role for the C-terminal domains *in vivo*. Although our results clearly show that the mouse has managed without full-length ADAMTS13, the relative importance of ADAMTS13 for regulation of VWF activity may be different between human and mouse. A gene targeting technique of mouse *Adamts13* will help to clarify the physiological contribution of mADAMTS13.

Acknowledgments—We thank Yuko Nobe, Yoko Tokunaga (National Cardiovascular Center Research Institute), Noriko Mimura, and Arisa Maeyashiki (The Chemo-Sero-Therapeutic Research Institute) for technical assistance.

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AJH 2004; 17:446–449

Three Novel Missense Mutations of *WNK4*, a Kinase Mutated in Inherited Hypertension, in Japanese Hypertensives

Implication of Clinical Phenotypes

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Background: Mutations in serine-threonine kinase *WNK4* with no lysine (K) at a key catalytic residue cause familial hypertension known as pseudohypoaldosteronism type II (PHAII). The objective of this study was to test whether more subtle changes of *WNK4* could be implicated in hypertension or renal failure.

Methods: We screened 956 Japanese patients with hypertension or renal failure for mutations in exons 7 and 17 in the *WNK4* gene where the mutations were identified in patients with PHAII.

Results: We identified three novel missense mutations, Met546Val (n = 2) and Pro556Thr (n = 2) in exon 7, and Pro1173Thr (n = 1) in exon 17, in a heterozygous state in addition to four single nucleotide polymorphisms including one synonymous mutation (Ala547Ala). Results of genotyping Met546Val and Pro556Thr mutations indicated that these mutations were not present in a Japanese general population (n = 1875).

Conclusions: The present study indicated that a systematic screening of *WNK4* in a large set of patients with hypertension or renal failure detected some rare genetic variants. Although substantial contribution of three novel missense mutations in exons 7 and 17 of *WNK4* to the genetics of hypertension or renal failure is still unclear, these mutations in the *WNK4* gene identified in Japanese hypertensives but not in a general population may contribute to hypertension and progression of hypertensive complications to some extent. Am J Hypertens 2004;17: 446–449 © 2004 American Journal of Hypertension, Ltd.

Key Words: Pseudohypoaldosteronism, *WNK4*, gene variants, hypertension.

Pseudohypoaldosteronism type II (PHAII), so-called Gordon syndrome,[1] is an autosomal dominant disease characterized by hypertension, hyperkalemia, normal renal glomerular filtration, and renal tubular acidosis caused by impaired renal K^+ and H^+ excretion. These physiologic abnormalities are all chloride dependent[2] and can be corrected by thiazide diuretics, specific antagonists of the Na-Cl co-transporter present at the distal convoluted tubule. It has been reported that mutations in either of two serine-threonine kinases, *WNK1* and *WNK4*, can cause PHAII.[3] The defect in the *WNK1* gene was a large deletion in intron 1, resulting in increased *WNK1* expression.[3]

Immunohistochemical analysis showed that *WNK1* is ubiquitously expressed in many tissues and organs,[4,5] and seems restricted to diverse chloride-transporting epithelia.[3,6] In the kidneys, *WNK1* is restricted to the aldosterone-sensitive distal nephron.[3,6]

WNK4 expression is predominantly limited to the distal convoluted tubule, connecting tubule, and collecting duct in the kidney.[3] In the distal convoluted tubule, *WNK4* co-localizes with XO-1, a specific tight junction protein, whereas in the cortical collecting duct, *WNK4* expression is mostly cytoplasmic, suggesting a specific function along the nephron axis.[3] Recent expression studies indicated that the co-expression of the thiazide receptor, the Na-Cl co-

Received July 22, 2003. First decision September 21, 2003. Accepted December 23, 2003.

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This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan.

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0895-7061/04/$30.00
doi:10.1016/j.amjhyper.2003.12.020