Association of eNOS Glu298Asp Polymorphism With End-Stage Renal Disease

Eisei Noiri, Hiroaki Satoh, Jun-ichi Taguchi, Sergey V. Brodsky, Akihide Nakao, Yumiko Ogawa, Satomi Nishijima, Takehiko Yokomizo, Katsushi Tokunaga, Toshiro Fujita

Abstract—Nitric oxide (NO) derived from endothelial cells is profoundly related to the maintenance of physiological vascular tone. Impairment of endothelial NO generation brought about by gene polymorphism is considered the major deterioration factor for progressive renal disease, including diabetic nephropathy. The present study aimed to elucidate the Glu298Asp polymorphism of endothelial NO synthase (eNOS) in patients with end-stage renal disease (ESRD) and its role as a predisposing factor for cardiovascular complications. Glu298Asp in exon 7 of the eNOS gene was determined by polymerase chain reaction, followed by restriction fragment length polymorphism analysis, in ESRD patients (n=185) and compared with that of unrelated healthy individuals (n=304). The occurrence of 298Asp was significantly higher in the ESRD group (P=0.0020; odds ratio [OR] 1.65; 95% confidence interval [CI]: 1.21 to 2.25). In this group, 72 patients had type 2 diabetes mellitus (DM). Although 298Asp did not reach a significant level in the non-DM ESRD subgroup, the occurrence of 298Asp was significantly higher in DM-derived ESRD patients (P=0.0010; OR 2.02; 95% CI: 1.37 to 3.07). The functional effect of the Glu298Asp was examined using Chinese hamster ovary (CHO) cells stably overexpressing either 1917G or 1917T. NO-selective electrode measurements and fluorometric nitrite assay revealed a statistically significant difference in NO production or nitrite accumulation between CHO 1917G and 1917T (P<0.01). These data indicated that Glu298Asp is the predisposing factor in ESRD, especially DM-derived ESRD. The functional difference in NO generation depending on eNOS with either glutamate or aspartate at position 298 was also confirmed in vitro. (Hypertension. 2002;40:535-540.)

Key Words: polymorphism ■ polymerase chain reaction ■ nitric oxide synthase ■ diabetes mellitus

The endothelial isoform of nitric oxide (NO) synthase (eNOS, NOSIII) is a constitutively expressed 135 kDa protein predominantly associated with the particulated specific structures in the plasmalemmal membrane, caveolae, of vascular endothelial cells.\(^1\)\(^2\) NO is produced from \(L\)-arginine and diffuses to vascular smooth muscle cells, where it increases the concentration of cGMP by stimulating soluble guanylate cyclase, leading to vascular relaxation. It also inhibits platelet and/or leukocyte adhesion to vascular endothelium. Therefore, the impairment of eNOS expression has been considered a primary factor for diseases such as hypertension, coronary artery disease, thromboembolic diseases, and atherosclerosis. Indeed, knocking out the gene encoding eNOS in mice resulted in significant hypertension, and aortic rings from these animals studied ex vivo displayed no relaxation in response to acetylcholine.\(^3\) Patients with end-stage renal disease (ESRD) derived from diabetes mellitus (DM) nephropathy have a higher prevalence of cardiovascular complications than those with non-DM ESRD, and this limits their 5-year survival rate to less than 50%. Therefore, polymorphism in eNOS is considered one of the major predisposing factors for endothelial dysfunction.

The gene polymorphisms of eNOS have been detected at 4b/4a variable number of tandem repeats in intron 4, Glu298Asp in exon 7, CA repeat in intron 13, A27 to C (A to C nucleotide conversion) in intron 18, and G10 to T in intron 23. A part of candidate variations (4b/4a tandem repeats in intron 4) has been investigated in glomerulonephritis and/or ESRD patients, but no conclusive results have been obtained so far. Recently, the G/T polymorphism in exon 7 coding for Glu298Asp was detected and reported to have a remarkable association with coronary spasm, acute myocardial infarction, and hypertension.\(^4\) To date, this is the only known eNOS polymorphism associated with an altered protein sequence, though recent expression studies have demonstrated no functional difference between 298Glu and 298Asp despite the accumulating clinical evidence.\(^6\)\(^7\)

Received January 28, 2002; first decision February 19, 2002; Revision accepted August 2, 2002.
From the Departments of Nephrology and Endocrinology (E.N., A.N., T.F.), Diabetes Mellitus and Metabolism (H.S.), Human Genetics (K.T.), and Biochemistry and Molecular Biology (T.Y.), University of Tokyo, Tokyo, Japan; Department of Cardiovascular Disease, Tokai University (J.T.), Kanagawa, Japan; Department of Medicine, State University of New York at Stony Brook (S.V.B.), Stony Brook, New York; and Molecular Medicine Laboratories, Yamanouchi Pharmaceutical Co, Ltd (Y.O., S.N.), Tsukuba, Japan.
Correspondence to Eisei Noiri, MD, PhD, Department of Nephrology and Endocrinology, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. E-mail noiri-tky@umin.ac.jp
H.S. and J.T. contributed equally this study.
© 2002 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org
DOI: 10.1161/01.HYP.0000033974.57407.82

535
Therefore, the purpose of the present study was to elucidate the Glu298Asp polymorphism in ESRD patients, to determine its role as a predisposing factor in the disease process, and to elucidate any alteration of eNOS enzymatic activity caused by this polymorphism.

Methods

Study Population

The Glu298Asp polymorphism in exon 7 of the human eNOS (heNOS) gene was determined in 185 patients with ESRD who were maintained on hemodialysis at 2 suburban dialysis centers in Tokyo, Japan, from 1996 to 1998. This polymorphism was then compared with that of 304 unrelated healthy individuals (control) who visited the urban health center in Tokyo, Japan, for routine health check-ups from 1997 to 1998. The average length of time from the onset of ESRD to the time of this study was 6.3 ± 5.4 (mean ± SD) years. All ESRD patients had creatinine clearance of < 10 mL/min and were treated 3 times a week with hemodialysis for 4 to 5 hours. In the ESRD group, 72 patients had type 2 DM. The remaining patients had chronic glomerulonephritis (n = 71; 3 of whom had previous renal transplantation), hypertensive nephrosclerosis (n = 22), polycystic kidney disease (n = 9), and miscellaneous conditions (n = 11). There were statistical differences between the 2 groups with respect to age (71.7 ± 12.3 years in the ESRD group versus 56.7 ± 10.2 years in the control group, mean ± SD, P < 0.01) and gender (male-to-female ratio, 136:49 in ESRD versus 195:110 in control, P < 0.01). In addition, in the ESRD group, the clinical data (eg, serum creatinine, serum urea nitrogen, serum uric acid, serum phosphate, serum potassium) reflected chronic renal failure, and SV1 + SV5 on the ECG was also significantly higher than that of the control group (P < 0.0001).

At the time of recruitment for this study, informed consent was obtained from each individual according to the protocol approved by the Human Study Committee of the University of Tokyo.

Genomic DNA

Genomic DNA from each subject in the study population was purified from peripheral blood leukocytes by SepaGene (Sanko-Junyaku) following the manufacturer’s protocol.

Genotyping

The detection of Glu298Asp polymorphism of the heNOS gene was performed following the previously reported protocol with minor modifications (detailed in an online supplement available at http://www.hypertensionaha.org). The typical pattern of electrophoresis was demonstrated in Figure 1A. All fragments uncleaved were double-checked by direct sequencing to avoid mistyping (Figure 1B).

The variable number of tandem repeats (VNTRs) in intron 4 of the eNOS gene (eNOS4a/b) was determined as previously described by Wang et al.

Direct Sequencing

Polymerase chain reaction (PCR) products were amplified, and both sense and antisense strands were directly sequenced using the same primer pairs as those for genotyping. Fluorescence-based automated cycle sequencing of PCR products was performed on ABI PRISM 310 (Perkin-Elmer) using a dye-terminator method according to the manufacturer’s instructions (dRhodamine Terminator Cycle Sequencing Kit; Perkin-Elmer).

Plasmid Constructs

Human wild-type–eNOS cDNA ligated into pcDNA3.1 expression vector (pcDNA3.1-WT-heNOS) (Invitrogen, Groningen, Netherlands) was constructed using a standard molecular protocol by Y.O. and S.N. The cDNA construct containing the 298Asp substitution was generated by site-directed mutagenesis using an LA PCR in vitro mutagenesis kit (Takara) and oligonucleotides, 5'-GCA GGC CCC AGA TGA TTC CCC AGA ACT CTT CC-3', according to the manufacturer’s instructions (detailed in the online supplement). The presence of the substitution and the entire sequence of the inserted fragment were verified by DNA sequencing.

Cell Culture and Stable Transfection

Chinese hamster ovary (CHO) cells were obtained from Riken Cell Bank (Wako) and grown in Ham F12 (Sigma) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). CHO-, wild-type- and mutant-type–heNOS cell clones were established by stable transfection with zeocin selectable pcDNA3.1 plasmid (Invitrogen). Transfection was performed using lipofectamine-plus (Gibco) according to the manufacturer’s instructions. Three days after transfection, cells were placed into the selection medium containing 1 mg/mL zeocin (Invitrogen). On day 14 after selection, individual zeocin-resistant colonies were subcloned.

Western and Southern Analysis

CHO cells (1 × 10⁵) were dissolved in the ice-cold extraction buffer and standard Western analysis was performed using monoclonal antibodies to heNOS (Transduction Laboratory) (detailed in the online supplement).

Genomic DNA of CHO cells was isolated by the Wizard genomic DNA purification kit (Promega) following the manufacturer’s protocol. Seven micrograms of genomic DNA was digested with one (Hind III; Takara) or no (ClaI, Takara) recognition site within the zeocin-selectable pcDNA3.1-heNOS plasmid. After electrophoresis on 1% agarose gel, transfer to nylon membrane (Hybond) was performed using SouthernMax reagents (Ambion) following the

Figure 1. Representative gel and sequencing analysis. A, PCR products were amplified from human genomic DNA and digested by restriction enzyme BanII. Homozygotes with G (Glu) to T (Asp) substitution at nucleotide position 1917 (TT) showed a single band at 457 bp (GG). Heterozygotes with G showed 2 bands at 320 bp and 137 bp. B, heNOS exon 7 was amplified from genomic DNA obtained from healthy individuals (left) and ESRD (right) and directly sequenced, which demonstrated a homozygous G to T transition at nucleotide position 1917.
manufacturer’s protocol. The DNA probe was labeled by alkaline phosphatase using AlkPhos Direct (Amersham). Chemiluminescent signal development was performed by CDP-Star (Amersham), and the signal was detected by Las-1000 (Fujifilm).

Fluorometric Nitrite Analysis
Nitrite production by CHO cells was measured by the fluorometric nitrite assay, which forms a fluorescent product of 1H-naphthotriazole reacting with nitrite. Fluorometric assay is about 50 to 100 times more sensitive than the Griess reaction, as shown in Figure 2A. Cells were seeded in 12-well plates (Corning) in DMEM and grown in 12-well plates (Corning) in DMEM/H11003 and incubated for 15 minutes at 37°C with 5% CO₂ and 95% air. The cell culture supernatant was centrifuged once at 1000 g/mL for 10 minutes at 4°C. The supernatant was withdrawn for determination of nitrite concentration. Simultaneously, the nitrite concentration was determined using a calibration curve with NaNO₂ as the standard. The aliquot was centrifuged once at 9000 g for 1 minute at 4°C, and the nitrite concentration was determined using a calibration curve with NaNO₂ as the standard.

Figure 2. Fluorometric nitrite assay. A, Standard curve. B, Nitrite measurement after addition of A23187. W indicates CHO 1917G cells; M, CHO 1917T cells; RFU, relative fluorescence unit. The measurements were performed 4 times in each set of triplicate experiments. The mean intensity of fluorescence in each group was used for the statistical analysis between W and M.

Statistical Analysis
The statistical difference in genotype distribution between the groups was examined by the Mantel-Haenszel test, considering confounding factors (eg, age and gender) between groups, the stratification of which is shown in Tables 1 to 3. Where appropriate, the common odds ratios (ORs) were calculated. Standard error by Robins-Greenland-Breslow, 95% confidential interval (CI), and probability confidence interval.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>ESRD</th>
<th>Control</th>
<th>ESRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA, n</td>
<td>64</td>
<td>24</td>
<td>58</td>
<td>27</td>
</tr>
<tr>
<td>Aa+aa, n</td>
<td>6</td>
<td>11</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>OR^</td>
<td>4.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50≤y&lt;60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA, n</td>
<td>92</td>
<td>34</td>
<td>86</td>
<td>35</td>
</tr>
<tr>
<td>Aa+aa, n</td>
<td>17</td>
<td>9</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>OR^</td>
<td>2.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60&lt;y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA, n</td>
<td>95</td>
<td>78</td>
<td>105</td>
<td>83</td>
</tr>
<tr>
<td>Aa+aa, n</td>
<td>30</td>
<td>29</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>

Results
Association Between ESRD and Polymorphism of heNOS Gene
The frequency of Glu298Asp (G1917T) in 185 ESRD patients and in 304 healthy individuals (controls) was compared (Table 1). The distribution of genotypes in the control group did not differ significantly from that expected under Hardy-Weinberg equilibrium. The positivity of 298Asp was significantly higher in the ESRD patients (P=0.0020). The OR for the ESRD group was 1.65 (95% CI: 1.21 to 2.25). On the other hand, the positivity of eNOS4a allele in the ESRD was not statistically different from the control group (P=0.187).

Association Between ESRD From Type 2 DM and Polymorphism of heNOS Gene
In the ESRD group, 72 patients had ESRD resulting from type 2 DM nephropathy. The frequency of Glu298Asp in this subgroup was compared with that of controls (Table 2). The
positivity of 298Asp was significantly higher in the patients with DM-derived ESRD \( (P < 0.001) \), and its OR was 2.02 (95% CI: 1.34 to 3.07). In contrast, there was no significant effect of this genotype on the incidence of ESRD without DM (Table 3).

**Expression of heNOS in Transfected CHO Cells**

To examine whether heNOS with glutamate or aspartate at position 298 influenced the level of NO production, CHO cells were stably transfected with 2 eukaryotic expression vector constructs, heNOS 1917G (W1, W2, W3, W6 in Figure 3) and 1917T (M2, M3, M6, M9), resulting in heNOS 298 glutamic acid and aspartic acid, respectively. Western blot analyses of heNOS in CHO 1917G and CHO 1917T are shown in Figure 3. No difference in heNOS protein expression was found among cells. Copy numbers of each construct were further examined by Southern analysis (data not shown), which confirmed Western analysis results.

**Functional Analysis of heNOS With or Without heNOS Polymorphism**

Calcium ionophore, A23187, initiated NO production by heNOS. This NO production was measured by both NO-selective electrode and fluorescent analysis. As shown in Figure 4, NO production from CHO 1917G detected by the NO-selective electrode was \( 188 \pm 18.8 \) nmol/L (n=10; mean±SE) compared with CHO 1917T of \( 56.1 \pm 9.4 \) nmol/L (n=9; \( P < 0.01 \)). Fluorescent nitrite detections from CHO 1917G or 1917T of \( 2 \times 10^5 \) cell population are shown as RFUs in Figure 2B, which was calculated as \( 4.33 \pm 0.030 \) mol/L and \( 3.89 \pm 0.20 \) mol/L (mean±SE; \( P < 0.01 \)), respectively, using a standard curve (Figure 2A).

**Discussion**

Endothelial dysfunction and premature atherosclerosis may have potential genetic factors that could accelerate to ESRD in chronic glomerulonephritis and DM nephropathy. The Glu298Asp polymorphism of heNOS needed to be evaluated to determine whether it is a major candidate gene for progressive renal disease, because significant accumulation of the Glu298Asp mutation in coronary artery disease,12 acute

---

**TABLE 2. Distribution of Genotype in Patients With ESRD Resulting From Type 2 DM and in Healthy Controls**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=304)</th>
<th>ESRD From Type 2 DM (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu/Glu, n</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>50≤y&lt;60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>13</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>60&lt;y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>95</td>
<td>32</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>3</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>OR (^a)</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>159</td>
<td>46</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>35</td>
<td>15</td>
</tr>
</tbody>
</table>

**TABLE 3. Distribution of Genotype in Patients with Non-DM ESRD and in Healthy Controls**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=304)</th>
<th>Non-DM ESRD (n=113)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu/Glu, n</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>OR (^a)</td>
<td>5.33</td>
<td></td>
</tr>
<tr>
<td>50≤y&lt;60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>21</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>60&lt;y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>95</td>
<td>46</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>92</td>
<td>29</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/Glu, n</td>
<td>159</td>
<td>58</td>
</tr>
<tr>
<td>Glu/Asp+Asp/Asp, n</td>
<td>35</td>
<td>17</td>
</tr>
</tbody>
</table>

\(^a\) OR that met only the criteria satisfying both \( P < 0.05 \) and CI lower limit of \( >1.0 \) is shown here.

NO-selective electrode was \( 188 \pm 18.8 \) nmol/L (n=10; mean±SE) compared with CHO 1917T of \( 56.1 \pm 9.4 \) nmol/L (n=9; \( P < 0.01 \)). Fluorescent nitrite detections from CHO 1917G or 1917T of \( 2 \times 10^5 \) cell population are shown as RFUs in Figure 2B, which was calculated as \( 4.33 \pm 0.030 \) mol/L and \( 3.89 \pm 0.20 \) mol/L (mean±SE; \( P < 0.01 \)), respectively, using a standard curve (Figure 2A).
myocardial infarction, essential hypertension, carotid atherosclerosis, and preeclampsia have recently been reported. Because Glu298Asp missense was found in exon 7, the functional relevance in altering endothelial vasodilatation was expected and indirectly examined. Philip et al injected the alpha-adrenergic agonist, phenylephrine, into patients undergoing cardiac surgery and found the enhanced responsiveness to alpha-adrenergic stimulation in patients with the 894T allele. Therefore, we examined the involvement of Glu298Asp missense in susceptibility to ESRD and constructed CHO cells stably overexpressing either 1917G or 1917T cells to elucidate functional consequences directly.

The relevance of polymorphism in heNOS to type 2 DM is expected after the recent analysis of linkage of type 2 DM nephropathy with the heNOS region on chromosome 7q in Pima Indians. The deletion of 1 of 5 nucleotide repeats in intron 4 of heNOS may be the best-studied candidate gene for diabetic nephropathy in both type 1 and type 2 DM. The level of mRNA and protein of heNOS between normal and variant alleles is equivocal because the intron may be excised during RNA processing. Therefore, it is expected that the mutation within introns could affect rates of heNOS transcription and/or processing rate, which may affect heNOS enzymatic activity. The significant association of this variation in intron 4 with diabetic nephropathy has recently been observed in both type 1 and type 2 diabetic nephropathy. Though Glu298Asp did not reach a statistically significant level in type 1 diabetic nephropathy, the deletion of 1 of 5 nucleotide repeats in intron 4 was also not statistically significant in type 2 diabetic nephropathy. The deletion of 1 of 5 nucleotide repeats in intron 4 was also not statistically significant in type 2 diabetic nephropathy with proliferative diabetic retinopathy. It was also insignificant in hypertension and ischemic heart disease in the Japanese population. In addition, Miyamato et al addressed the significant linkage disequilibrium between eNOS4a/b and G to T conversion in intron 23, which is the polymorphism not related to essential hypertension as reported by Bonnardeux et al. No significant linkage disequilibrium between eNOS4a/b and Glu298Asp (P=0.773, χ²=0.083), as well as no significant association of eNOS4a allele with ESRD, was observed in the present study.

The present study, in which Glu298Asp polymorphism in ESRD and type 2 diabetic nephropathy associated with it was found for the first time, demonstrated a significant increase of 298Asp in ESRD patients. It also revealed significant accumulation of this mutation predominantly in patients with ESRD resulting from type 2 DM, compared with patients with non-DM ESRD. These observations point to 298Asp not only as the candidate gene of progressive renal disease, but also as a possible prognostic factor in patients with DM-derived ESRD, because the 5-year survival rate of these patients is significantly shorter than that of patients with non-DM ESRD. One of the major reasons for this poor prognosis is that the prevalence of atherosclerotic complicating diseases, such as cerebrovascular accidents, coronary artery disease, and peripheral vascular disease, is much higher in the DM-ESRD population. Lesser NO production in 298Asp individuals may increase the proinflammatory leukocyte adhesion to endothelial cells and may decrease the vasodilatory capability, which could further exacerbate atherosclerotic lesions in these DM-ESRD patients with 298Asp.

The 3D representation of heNOS dimer demonstrates position 298 of amino acid at the exterior of the dimer surface, which apparently is not close to the catalytic site or the cofactor binding domains of heNOS. One possible explanation for its location may relate to the modification of protein-protein interaction, especially with caveolae, though the specific binding interaction of heNOS with endothelial membrane structures is not well defined. Another possible mechanism of functional alteration may be the stability of the heNOS enzyme and/or interactions with other parts of heNOS activity.

Tesauro et al and Fairchild et al recently investigated the difference in NO generation between heNOS with glutamate and aspartate at position 298, using a transient expression system of COS7 cells, and found no difference. However, it was reported that 298Asp was more vulnerable to enzymatic cleavage in cell lysates compared with Glu298 protein. The discrepancy in findings between those data and ours could be attributed to the differences in copy numbers expressed in cells and the proper expression of proteins at high levels, which are difficult to adjust in a transient transfection system, as well as to the sensitivity of methods used to measure NO generation. Because in our study the CHO cells with mutation showed a lower level of NO generation, it would be reasonable to compare NO production after performing both the adjustment of copy numbers and the confirmation of heNOS protein levels.

In the present study, CHO cells with 298Asp showed a measurable amount of NO, which suggests that this polymorphism does not impair NO production in an all-or-nothing manner. However, the graded differences in NO generation could play a much more critical role in the pathophysiological condition at the microvascular level. Conservation of the vasodilatory potential in eNOS-deficiency has been attributed to the upregulation of other NOS isoforms and the induction of vasodilatory prostaglandins or endothelium-derived hyperpolarizing factor. However, 298Asp mutation does not produce a phenotype of null mutation and, therefore, should have a lesser effect on other vasodilatory mediators.

**Perspectives**

The observed accumulation of 298Asp, especially in patients with DM as a cause of ESRD, suggests that this polymorphism is the candidate factor for the accelerated nephrop-
athy in type 2 DM. In addition, the accumulation of 298Asp may contribute to the poor prognosis for the 5-year survival rate in DM-ESRD patients compared with that of other ESRD patients. This inference is logical, given that, in Japan, the principal life-threatening complication of type 2 DM in ESRD is ischemic heart disease, which has been remarkably associated with 298Asp.4 This mutation is becoming more important because DM nephropathy has become the principal cause of the initiation of elective hemodialysis in Japan.28 To elucidate this possibility, a prospective study based on hNOS polymorphism is needed. In future studies, it is also certainly necessary to investigate the association of the current polymorphism with other variants and/or a specific haplotype, which should provide a more comprehensive analysis of the eNOS gene. [Author: McMillan et al] was not cited in the article.]

Acknowledgments

The authors are grateful to Dr Michael S. Goligorsky (M.S.G.) for his critical reading of the manuscript. These studies were supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.N., K.T.), Jisme-Hinketsu Kenkyukai Foundation (Japan: E.N., A.N.), and Takeda Science Foundation (Japan: E.N.), as well as by grant support from M.S.G. Foundation (Japan: E.N.), as well as by grant support from M.S.G.

References


26. Huang A, Sun D, Smith CJ, Connetta JA, Shesely EG, Koller A, Kaley G. In eNOS knockout mice skeletal muscle arteriolar dilatation to acetyl-


Association of eNOS Glu298Asp Polymorphism With End-Stage Renal Disease
Eisei Noiri, Hiroaki Satoh, Jun-ichi Taguchi, Sergey V. Brodsky, Akihide Nakao, Yumiko Ogawa, Satomi Nishijima, Takehiko Yokomizo, Katsushi Tokunaga and Toshiro Fujita

Hypertension. 2002;40:535-540; originally published online September 3, 2002;
doi: 10.1161/01.HYP.0000033974.57407.82

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/40/4/535

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2002/10/02/40.4.535.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/