

Effects of Glu298Asp Polymorphism of Endothelial Nitric Oxide Synthase (eNOS) Gene on eNOS mRNA and Protein Expressions in Cultured Human Vascular Endothelial Cells

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The relationship between cardiovascular disease and Glu298Asp polymorphism of nitric oxide synthase (eNOS) gene in vascular endothelial cells is controversial due to conflicting results. To demonstrate the effects of Glu298Asp polymorphism of the eNOS gene on eNOS gene expression by excluding the effects of various confounding factors, eNOS mRNA and protein expressions were measured using cultured human umbilical vein endothelial cells (HUVECs) according to genotypes of Glu298Asp polymorphism of the eNOS gene. eNOS mRNA expression was measured by real-time PCR/reverse transcription-PCR. eNOS protein expression was measured by an immunoassay method. HUVECs with Glu298Asp tended to show higher eNOS mRNA and protein expressions than those with Glu298Glu, but the difference was not significant. These results suggest that the genotypic difference of Glu298Glu and Glu298Asp has no effect on eNOS mRNA and protein expressions.

Key words: gene expression; nitric oxide synthase; polymorphism; vascular endothelial cell

Nitric oxide produced from vascular endothelial cells is synthesized from L-arginine by the functions of nitric oxide synthase (eNOS). Major functions of vascular endothelial cell-derived NO include relaxant and antiproliferative effects on vascular smooth-muscle cells, antiaggregant effects on the platelets, and inhibitory effects on adhesion of leukocytes to vascular endothelial cells (Kader et al., 2000; Ozaki et al., 2002). In addition, the relationship between NO production in vascular endothelial cells and various pathologic conditions such as inflammatory disease, hypertension, and coronary artery disease has been reported.

The eNOS gene is located on chromosome 7

(7q35-36), spans 21 kb, and consists of 26 exons (Marsden et al., 1993). Among several reported polymorphisms of the eNOS gene, the involvement of Glu298Asp polymorphism (glutamic acid to aspartic acid substitution at codon 298) in coronary artery disease and essential hypertension has been discussed (Miyamoto et al., 1998; Kato et al., 1999; Shoji et al., 2000; Benjafeld et al., 2000; Colombo et al., 2002). The conflicting results are considered to be caused by confounding factors such as differences in the genetic backgrounds of study populations and various environmental factors.

It is important to experimentally investigate the functional effects of Glu298Asp polymor-

Abbreviations: CTPP, confronting 2-pair primers for polymorphism; eNOS, nitric oxide synthase; HUVEC, human umbilical vein endothelial cell

phism on eNOS gene expression by excluding these confounding factors. Thus, we identified Glu298Asp polymorphism using cultured human vascular endothelial cells, and investigated the functional effects of Glu298Asp polymorphism on eNOS mRNA and protein expressions.

Materials and Methods

Ethics

This study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Tottori University. Biomaterials (human umbilical cords) were collected after obtaining informed consent from the pregnant women.

Cell culture

Human umbilical cords were aseptically obtained immediately after delivery, and human umbilical vein endothelial cells (HUVECs) were collected by enzyme treatment with 0.5 g/L collagenase type IV (Wako Pure Chemical, Osaka, Japan). The collected HUVECs were plated onto gelatin-coated plastic culture dishes, and were statically cultured in a CO₂ incubator (5% CO₂ in 95% humidified air). An Endothelial Cell Growth Medium 2 Kit (Promo Cell, Heidelberg, Germany) was used as the growth medium. The kit includes the following: endothelial cell basal medium, 2% fetal calf serum, 5.0 ng/mL epidermal growth factor, 0.2 µg/mL hydrocortisone, 5.0 ng/mL vascular endothelial growth factor, 10 ng/mL basic fibroblast factor, 20 ng/mL R3 insulin-like growth factor-1, 1 µg/mL ascorbic acid, 22.5 µg/mL heparin, 50 ng/mL amphotericin B and 50 µg/mL gentamicin.

Identification of Glu298Asp polymorphism

DNA was extracted from umbilical cord tissue using an automatic nucleic acid extraction instrument (MFX-2000, Toyobo, Osaka), and a PCR

with confronting 2-pair primers for polymorphism (PCR-CTPP) method (Hamajima et al., 2000) was performed using 4 primers (primer 1: 5'-CAT GAG GCT CAG CCC CAG AAC-3', primer 2: 5'-AGT CAA TCC CTT TGG TGC TCAC-3', primer 3: 5'-GAA GGA AGA GTT CTG GGG GA-3', primer 4: 5'-GCT GCA GGC CCC AGA TGA G-3'). In the primary cultures of 29 cases, polymorphisms were identified. As a result, 26 cases with the Glu298Glu genotype and 3 cases with the Glu298Asp genotype were identified.

Comparative quantification of eNOS gene mRNA expression

mRNA was extracted from HUVECs using ISOGEN (Nippon Gene, Toyama, Japan). eNOS mRNA expression was measured by a real-time PCR/reverse transcription-PCR method. A Fast Start DNA Master SYBER Green I Kit (Roche Diagnostics, Tokyo, Japan) was used as a reagent, and the primers used were as follows: 5'-CAT GAG GCT CAG CCC CAG AAC-3' and 5'-AGT CAA TCC CTT TGG TGC TCAC-3'. A Takara PCR Thermal Cycler (Takara Bio, Otsu, Japan) was used for cDNA synthesis under the following reaction conditions: annealing at 30°C for 10 min, reverse transcription at 42°C for 15 min, and inactivation of reverse transcriptase at 99°C for 5 min. A LightCycler (Roche Diagnostics) was used for real-time PCR. PCR was performed under the following conditions: after denaturing at 95°C for 10 min, 50 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 5 s, and extension at 72°C for 10 s with a temperature transition rate of 20 °C/s. Fluorescence was recorded following the extension reaction stages of each cycle. β-actin gene mRNA expression was also measured for comparative quantification, and eNOS and β-actin mRNA expressions were quantified comparatively by calculating the ratio between them. The primers used for β-actin gene were as follows: 5'-CGT GAC ATT AAG GAG AAG CTG TGC-3' and 5'-GTC ATA CTC CTG CTT GOT GAT CCA-3'.

Quantification of eNOS protein expression

An immunoassay method by a Human eNOS Immunoassay Kit (R&D Systems, Minneapolis, MN) was used to measure the eNOS protein expression. In brief, cells were trypsinized for collection, and were precipitated by centrifugation at $300 \times g$ for 5 min. Then cells were washed twice with phosphate-buffered saline, and after the addition of cell lysis buffer, the cell solution was centrifuged at $300 \times g$ for 5 min, and the concentration of eNOS protein in the supernatant was measured by a microplate reader.

Experiments according to Glu298Asp genotypes

HUVECs with the Glu298Glu or Glu298Asp genotype were plated onto 2 gelatin-coated 60 mm plastic dishes, and were cultured in growth medium to reach confluence. Subsequently, the medium was replaced with fresh growth medium, and 24 h later, mRNA and protein expressions were measured as described above. Measurement was conducted on different dates using different HUVECs derived from 5 and 3 individuals for Glu298Glu and Glu298Asp genotypes, respectively.

Statistical analysis

SPSS software (version 11.0J) was used for statistical analysis. Student's *t*-test was used as a statistical method for comparison. The significance level was set to 5%.

Results

The effects of genotypes on eNOS mRNA and protein expressions are shown in Fig. 1. eNOS mRNA expression tended to be higher in HUVECs with the Glu298Asp genotype than in those with Glu298Glu (Fig. 1A), but the difference was not significant. Also, eNOS protein

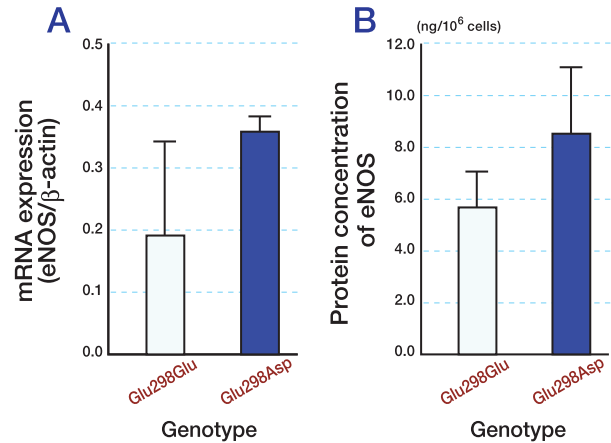


Fig. 1. eNOS mRNA expression (A) and protein concentrations (B) according to genotypes of eNOS polymorphism Glu298Asp in exon 7. Results are expressed as means (SE; bars). Values for eNOS mRNA expression (A) are given as the ratio of the amounts of eNOS and β -actin mRNA expression measured by real time PCR/reverse transcription-PCR. Values for eNOS protein (B) are measured by immunoassay method. Measurement was conducted on different dates using different HUVECs derived from 5 and 3 individuals for Glu298Glu and Glu298Asp genotypes, respectively.

expression tended to be higher in HUVECs with the Glu298Asp genotype than in those with Glu298Glu (Fig. 1B), but the difference was not significant.

Discussion

To reveal the effects of Glu298Asp polymorphism of the eNOS gene on eNOS mRNA and protein expressions, eNOS mRNA and protein expressions were measured using HUVECs with the Glu298Glu or Glu298Asp genotype. Both eNOS mRNA and protein expressions tended to be higher in HUVECs with Glu298Asp than in those with Glu298Glu, but the difference was not significant.

Mainly in case-control studies, the relationship between the Glu298Asp polymorphism of the eNOS gene and cardiovascular disease is controversial due to conflicting results (Miyamoto

et al., 1998; Kato et al., 1999; Shoji et al., 2000; Benjafield et al., 2000; Colombo et al., 2002). We conducted a cohort study on the relationship between the Glu298Asp polymorphism of the eNOS gene and hypertension in Japanese, but found no relationship (Kishimoto et al., 2004).

Also, there are several studies on the effects of the eNOS Glu298Asp genotype on the functions of the circulatory system. Schneider et al. (2000) investigated the dilation reaction of the forearm artery by administering acetylcholine to 80 patients, and reported no difference due to the eNOS Glu298Asp polymorphism. Jesung et al. measured plasma nitric oxide levels derived from vascular endothelial cells, and reported no difference due to the eNOS Glu298Asp polymorphism. Our study supports these results, showing no effects of the Glu298Asp polymorphism of the eNOS gene on the circulatory system.

This study was limited by the unavailability of HUVECs with the homozygotic Asp298Asp genotype. Our cohort study included 85.3% Glu298Glu, 14.0% Glu298Asp and 0.7% Asp298Asp ($n = 2,042$). It is considered necessary to obtain HUVECs with the homozygotic Asp298Asp genotype by increasing the sample size in the future.

In conclusion, the results of this study suggest the possibility that the Glu298Glu and Glu298Asp polymorphisms of the eNOS gene have no effect on eNOS mRNA and protein expressions in HUVECs, and also on the circulatory system.

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