Original Article

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

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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-
showed increased expression compared to the 3A allele (16) reporter gene, Popowski in the cancer or, alternatively, whether it is linked to another variant Lys198Asn polymorphism by itself has functional significance, and the Asn-allele has been associated with raised systolic BP (13). 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, or with 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% CO2 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 EcoRI and SalI 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'-CACGCTGAAAGGCAATGAGCTCTCTGGAGGGATT-3' and reverse 5'-CGCTCTGGAGGAATTGGCCTTTCAGCTTG-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 1μg of expression plasmid (pCI/hET-1/Lys, pCI/hET-1/Asn or pCI) and 5ng of pMI-SEAP (Roche

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.
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 42 h, and then the culture supernatant was collected. The transfected cells were incubated for another 42 h, and then the culture supernatant was collected. The amounts of immuno reactive ET-1 and big ET-1 were measured by enzyme linked immunosorbent assay (ELISA) using the Endothelin-1 Measurement Kit-IBL and Human Big Endothelin-1 Measurement Kit-IBL, respectively (Immu no-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 levels 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-AGGCCAGCCCTCCA-MGB (Lys-allele), Vic-AGGGCAATCCCTCCAG-MGB (Asn-allele), and primers 5 IPPAGGC CTCACATGTCTTCTTGT-3 ′ and 5 GTCCTCAAGTGAC GCCCAATG TGCTCGGTGT-3 ′ for 3A/4A, probes Fam-AGTGCC CTTTACACGG-MGB (4A-allele) and Vic-AAGGCT TTAACCGG-MGB (3A-allele), and primers 5 AAACACGC TTCCAGTCCCTCAA-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 SNPAllyze version 3.0 software (DYNACOM, Mobra na, 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, proproET-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/m²; mean BP: 144.5±7.3/82.2±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).
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 retained from Japanese hypertensives. LD analysis revealed that the Lys198Asn and 3A/4A polymorphisms are slightly retained from Japanese hypertensives. 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.

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References