Correlation Between Intron 4 Polymorphism of the Endothelial Nitric Oxide Synthase Gene and Cardiovascular Risk with the Numbers of Circulating Endothelial Progenitor Cells in Healthy Subjects

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Abstract In the present study, we investigated the relationship between an important 27 bp repeat polymorphism in intron 4 of eNOS and numbers of circulating EPCs in presence of cardiovascular disease (CVD) risk factors in a group of healthy human volunteers. The study comprised of 45 healthy subjects (30–50 years). These subjects had various degrees of CVD risk but no history of CVD. The repeat polymorphism of eNOS was detected by polymerase chain reaction and EPC levels were analyzed by flow cytometry. We observed a good association between the intronic 4 mutant a/b genotype and the combined Framingham risk factor score in our subjects ($\chi^2 = 3.2$, $P = 0.07$). EPC numbers in subjects with mutant eNOS a/b genotype were also less than those observed in subjects with normal eNOS b/b genotype ($P = 0.06$). Interestingly, subjects with eNOS a/b genotype showed a significant inverse correlation between framingham risk score and EPC numbers ($R = -0.57$, $P < 0.05$). The study suggests that the presence of CVD risk factors in subjects with eNOS intron 4 polymorphism results in reduced number of circulating EPCs, which may significantly predispose them to CVD and aberrant endothelial repair.

Keywords Cardiovascular disease · Endothelial nitric oxide synthase · Endothelial progenitor cells · Polymorphism

Introduction

Bone marrow-derived endothelial progenitor cells (EPCs) are known to play an important role in endogenous repair mechanism of the endothelial monolayer [1]. EPCs are mobilized into the peripheral blood from the bone marrow in response to stimulating signals from tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells contributing to both re-endothelialisation and neo-vascularisation [1–3]. Several studies including ours have reported an inverse correlation between the number, migratory activity and function of circulating EPCs and risk factors for cardiovascular disease (CVD) in healthy controls and CVD patients [4, 5]. Low levels of EPCs lead to attenuated vascular capacity in these subjects resulting in increased susceptibility to CVD [6, 7]. The ability of these cells for clinical use is also limited by their quantitative and qualitative impairment in subjects with CVD risk factors [7].

The major molecule that controls the migration and angiogenic functions of bone marrow-derived EPCs to the site of injury is eNOS-derived NO [8]. Endothelial NO synthase (eNOS) has been shown to elicit multiple beneficial effects within the cardiovascular system. eNOS catalyzes the conversion of L-arginine to L-citrulline, producing nitric oxide (NO) that plays a vital role in the regulation of vascular homeostasis. The endothelial nitric oxide synthase (eNOS) gene is located on chromosome 7q35-36. Several gene polymorphisms have been detected in the eNOS gene and evidences strongly suggest that bioavailability of eNOS-derived NO and thus endothelial function is linked to eNOS polymorphisms [9]. One of the major polymorphisms, present in intron 4 of eNOS, is a variable number of tandem repeats (VNTR) polymorphism giving rise to two alleles, namely allele ‘a’ that corresponds

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to four repeats of a 27 bp fragment and allele 'b' containing five repeats [10].

It is although well known that low EPC numbers and polymorphisms of eNOS are significantly associated with CVD risk factors, there are no studies focusing on the correlation, if any, between these polymorphism and EPC numbers. Since, the migration of EPCs from the bone marrow largely depends on eNOS, in the present study we investigated the hypothesis that subjects with low expression of eNOS owing to the presence of intron 4 eNOS polymorphism would have decreased number of EPCs in circulation.

Materials and Methods

Study Population and CVD Risk Factor Assessment

Forty-five healthy volunteers were included in the study, some of whom had conventional cardiovascular risk factors. The age of the subjects was in the range 30–50 years. Subjects were excluded from this study if they had known or symptomatic CVD or had any condition, such as cancer or retinopathy, in which neo-vascularization might be present. None of the controls were on any CVD medication. All the subjects underwent a detailed assessment of cardiovascular risk with informed consent. Risk factors such as hypertension, smoking, high glucose levels and hypercholesterolemia in the study group were collected and defined as previously described [5]. The total burden of risk factors was calculated online with use of the Framingham risk factor (www.cvdriskchecksecure.com/framinghamriskscore.aspx), which has previously been used to predict the risk of coronary artery disease in persons free of clinical disease [11]. All the measurements were performed following international guidelines. Both internal and external quality assessments were regularly done to ensure accurate and consistent results. The study was approved by the Human Ethics committee of Gautam Buddha University.

DNA Extraction and Genotyping of eNOS Intronic Polymorphism

A 5 ml blood sample was used for genomic DNA isolation by salting out procedure [12]. For the detection of eNOS intron 4 repeat polymorphism, PCR amplification of the eNOS intron 427 bp repeat was done using the primer pair: sense 5'-AGGCCCTATGGTAGTGCCTTT-3', antisense 5'-TCTCTTAGTGCTGTGGTCAC-3'. The PCR cycle consisted of an initial denaturation of 10 min at 94 °C, followed by 32 cycles of 94 °C denaturation for 45 s, 59.4 °C annealing for 1 min and 72 °C extension of 1 min and 10 s [13]. The final extension was carried out at 72 °C

for 10 min. The PCR amplicon was run on 2.7% agarose gel and observed under UV transillumination of the gel doc to detect the genotype of the subject. The 'b' allele is 420 bp corresponding to five repeats of a 27 bp fragment and the allele 'a' is 393 bp containing four repeats of the same fragment. PCR was repeated for some of the samples (n = 20) to ensure accuracy and consistency.

Flow Cytometry

For the enumeration of circulating EPCs, 50 µL of whole blood was taken in a FACS tube and incubated in dark for 20 min at room temperature with CD34-FITC and CD133-APC [14]. The fluorescent labelled sample was vortexed with 450 µL of 1× BD FACS lysing solution and incubated at room temperature for 10 min followed by washing with 1 ml 1X PBS. The cells were then acquired on a BD-FACS Calibur flow cytometer. For each sample, a minimum of 50,000 events was acquired. Lymphocytes were gated in linear FSC/SSC plot and EPCs were quantified and enumerated in this gate as percentage of dual positive (CD34–CD133) cells by BD CellQuest Pro software in all the subjects.

Statistical Analysis

Data are expressed as mean ± SD. Comparison of the EPC numbers between different control groups was analyzed by Student 't' test (2 sided). Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed versus predicted genotype frequencies. Pearson's χ^2 and *P* values for eNOS intron 4 genotypes with and without the presence of CVD risk factors in the study group was calculated using online χ^2 test. All analysis was done using SPSS (ver 16) and SISA (<http://home.clara.net/sisa/>). Statistical significance was assumed if a null hypothesis could be rejected at *P* < 0.05.

Results

Clinical Characteristics and eNOS Genotype of the Study Group

The clinical characteristics, eNOS genotype and EPC numbers of the study subjects are summarized in Table 1. Agarose gel image of the PCR amplicons show the presence of two different genotypes, b/b and a/b, in the study population (Fig. 1). No sample was found to have the homozygous a/a genotype in our study group. The percentage frequency of the other two genotypes, bb and ab in the study population was 66.7 and 33.3% respectively and

Table 1 Baseline characteristics of the study population

Characteristics	Study group (n = 45)
Ages (years)	33.8 ± 5.5
Percentage of current smokers	22.2%
Blood pressure (mmHg)	125.8 ± 13.4/82.5 ± 10.3
Total cholesterol (mmol/L)	4.4 ± 0.5
HDL (mmol/L)	1.2 ± 0.13
Blood glucose (mg/dL)	114.3 ± 18.2
Body mass index (BMI)	23.0 ± 3.3
EPC numbers	1.0 ± 0.8 (%)
Framingham risk score	3.6 ± 3.2
Percentage of mutant a/b genotype	33%

Values are given as mean ± SD

the percentage frequency of alleles, a and b was 83 and 17% respectively. The genotype frequencies were in agreement with Hardy–Weinberg expectations.

eNOS Genotype and CVD Risk Factors

The smokers in the study group had a higher frequency of the mutant a/b genotype as compared to the subjects with normal HDL ($\chi^2 = 2.7$, $P = 0.09$). All other risk factors didn't show an obvious correlation with the eNOS a/b genotype. Overall, subjects with high framingham risk score (score ≥ 3) showed a trend for greater presence of the mutant a/b genotype than the wild type genotype ($\chi^2 = 3.2$, $P = 0.07$).

EPC Numbers and eNOS Genotype

Since eNOS expression is closely associated with intron 4 VNTR SNP in the eNOS gene and eNOS-dependent

pathway plays a pivotal role in the process of EPC mobilization, migration and proliferation, we next determined the correlation of eNOS intron 4 SNP with circulating EPC numbers. To evaluate the percentage of circulating EPCs in the study subjects, flow cytometric analysis of the whole blood was performed. Cells positive for dual markers: CD34 and CD133 were counted as EPCs. Cells without any antibodies were used as negative controls (Fig. 2a, b). The average number of EPCs in subjects with a/b genotype was less than that present in subjects with b/b genotype ($0.75 \pm 0.5\%$ for a/b vs. $1.17 \pm 0.9\%$ for b/b, $P = 0.06$, Fig. 2c).

EPC Numbers, eNOS Genotype and CVD Risk Factors

EPC numbers have been inversely correlated with cardiovascular risk score. To assess the combined effect of eNOS genotype and cardiovascular risk score, we next studied the correlation of CVD risk factors with EPC numbers separately in subjects with b/b and a/b eNOS genotype. It was observed that subjects with eNOS a/b genotype showed a significant inverse correlation between framingham risk score and EPC numbers as compared to the subjects with eNOS b/b genotype (Fig. 3a, b, $R = -0.57$, $P < 0.05$ vs. $r = 0.006$, $P = \text{NS}$).

Discussion

The current study is the first one to address the role of eNOS gene polymorphisms in relation to EPC numbers in circulation. Among the various known SNPs of eNOS, the 27 bp repeat polymorphism in intron 4 of the gene is quite well known to negatively regulate the expression of eNOS

Fig. 1 Analysis of eNOS-PCR samples on 2% agarose gel. Wild type 'b' allele is 420 bp corresponding to five repeats of a 27 bp fragment and the mutant allele 'a' is 393 bp containing four repeats of the same

eNOS genotype	b/b	a/b
No. of subjects	N=30	N=15
Percentage	66.7	33.3

fragment. Individuals having the ab genotype show the presence of both the bands

Fig. 2 Flow cytometric analysis of the percentage of cell population expressing double antigens (CD133+ and CD34+) in **a** subject with b/b, **b** subject with a/b. Cells without the secondary antibodies were used as negative controls. **c** Box plots showing the levels of EPCs in subjects with eNOS genotype b/b and a/b. Values are represented by percentage (Mean $\pm$ SD)

Fig. 3 Linear regression analysis of percentage of EPCs with percentage framingham risk score in subjects with **a** eNOS genotype: b/b, **b** eNOS genotype: a/b. 'R' denotes regression co-efficient

via the production of 27 nucleotide (nt) sir-RNA [15]. More the number of 27-nt repeats, more 27 nt sir-RNA is produced and hence reduced expression of eNOS. Although this polymorphism is not an independent predictor of CVD, it has been associated with the presence of various CVD risk factors including smoking, hypertension, insulin resistant state and type 2 diabetes mellitus in

patients with CVD [16]. In the current study, we analyzed the association of this polymorphism with individual CVD risk factors and also with the total Framingham score in a group of healthy subjects. A high Framingham score is an indicator of a high cumulative risk for CVD [10]. The results demonstrated that the smokers showed a significant association with this genotype corroborating other studies

which suggest that smoking is a well-known factor inhibiting the release of physiological amounts of nitric oxide produced by endothelial nitric oxide synthase [6]. Overall, subjects with high Framingham risk score had higher frequency of the mutant a/b genotype than the subjects with low risk score.

Next, we investigated the correlation of eNOS genotype with numbers of EPCs in circulation. EPCs are believed to reside within the bone marrow in close contact with surrounding stromal cells. The migration of EPCs in circulation to the sites of injury has been reported to be largely dependent on eNOS activity and NO-mediated Akt signaling. Mice deficient in eNOS has been reported to display impaired ischemia-induced angiogenesis due to reduced EPC mobilization [17]. Most of the CVD risk factors are known to influence the mobilization or half-life of EPCs by modulating the levels of oxidative stress and having an inhibitory effect on eNOS activity [6, 18]. NO is also a significant regulator of neo-vascularization. In our previous studies, we have documented that an over-expression of eNOS gene in ex vivo cultured EPCs significantly increase their angiogenic properties both in vitro and in vivo [19, 20]. Although eNOS is documented to be a very important molecule governing the function and migration of EPCs, and eNOS expression is known to be controlled by the presence of eNOS gene polymorphisms, the correlation between EPC numbers and eNOS SNPs remains completely unknown. In the present study, subjects with mutant eNOS a/b genotype showed decreased EPC numbers as compared to the subjects with wild type b/b genotype, however this difference didn't attain any statistical significance, suggesting that this SNP may not be having a substantial effect on the number of circulating EPCs. However, the subjects with eNOS mutant a/b genotype showed significant inverse correlation between CVD risk score and reduced levels of EPCs in comparison to those having eNOS wild type b/b genotype, indicating that a combinatorial effect of defective eNOS genotype and CVD risk factors may exert a more detrimental effect on the EPC numbers. This is in coherence with a few other studies which show that eNOS gene defect may enhance vascular injury in presence of CVD risk factors [21, 22]. Small sample size is an obvious limitation of the study, however, an important conclusion drawn out from the study is that both genetic and environmental factors may govern the mobilization of EPCs in circulation and hence affect EPC-mediated repair in conditions of vascular injury. The study provides a novel hypothesis that in the presence of CVD risk factors, EPC levels in circulation may be genetically determined by eNOS intron 4 SNP. It would be worthwhile to validate our results in a larger study sample and also to determine how this gene-environment interaction affects the functional properties of

circulating EPCs, both in vitro and in vivo and thus the pathogenesis of CVD.

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Compliance with Ethical Standards

Conflict of interest All authors declare no conflict of interests.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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