Journal of Clinical Laboratory Analysis 19:87–92 (2005)

Distribution of Oxidation Enzyme eNOS and Myeloperoxidase in Primary Open Angle Glaucoma

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Genetic factors and the influence of superoxide are known to play roles in the etiology of glaucoma. We evaluated the association between primary open angle glaucoma (POAG) and two polymorphisms in the epithelial nitric oxide synthase (eNOS) gene, and one polymorphism in the myeloperoxidase (MPO) gene. We enrolled 66 patients with POAG and 100 healthy volunteers in this study. The polymorphisms in the eNOS and the polymorphism MPO −463 G-to-A in the MPO gene were resolved by polymorphism polymerase chain reaction (PCR). There were no significant differences in the distribution of the eNOS intron −4 ($P = 0.481$), eNOS promotor −786 (P=0.555), and MPO −463 (P=0.292) gene polymorphisms between the POAG patients and the volunteers (P-values=0.481, 0.555, and 0.292, respectively). None of the alleles from either gene differed between the groups (P-values=0.483, 0.554, and 0.183, respectively). Superoxide is closely related to glaucoma, and eNOS and MPO are two important enzymes in the free radical pathway. However, polymorphisms of the eNOS intron-4, eNOS promotor −786, and MPO −463 gene polymorphisms did not reveal significant differences between POAG patients and controls in our study. The use of these agents and other superoxide-related genes for clinical applications requires further investigation. J. Clin. Lab. Anal. 19:87–92, 2005.

Key words: endothelial nitric oxide synthase (eNOS); free radicals; myeloperoxidase (MPO); oxidation; primary open angle glaucoma (POAG)

INTRODUCTION

Glaucoma is a complex disease. In the past, mechanical and vascular effects were highly suspected as the mechanisms of this disease. Many recent studies have demonstrated that antioxidative agents are able to complement the protective effects of brain-derived neurotrophins against elevated IOP-induced ganglion cell damage, suggesting a potential role of oxidation in retinal toxicity (1,2).

Nitric oxide (NO) is a free radical and can react with reactive oxygen species to yield more cytotoxic species, including peroxynitrite anion (3,4). NO is also an active biological agent that is involved in the regulation of diverse physiological and pathological processes. It is synthesized from the amino acid L-arginine by NO synthase (NOS). NOS exists as three different isoenzymes: endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS) (3,4). eNOS and nNOS are expressed constitutively and release small amounts of NO. iNOS produces large amounts of NO in response to activation of cells by cytokines.

NO mediates neurotransmission and vasodilation (5). In the eye, NO regulates the baseline ocular blood flow rate in response to physiological stress, while suppression of NO production reduces the choroidal blood flow

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Received 20 August 2004; Accepted 9 November 2004
DOI 10.1002/jcla.20057
Published online in Wiley InterScience (www.interscience.wiley.com).

(6,7). A number of studies have indirectly shown that ocular NOS activity increases under physiological stress, such as ischemia and IOP elevation (8,9). In addition, NO has many diverse effects on living cells (10). Its inherent free radical nature may cause excitotoxic/oxidative damage to the eye (11). When NO combines with a superoxide anion (O_2^-), it results in a potent and a long-lived oxidant peroxynitrite anion ($ONOO^-$). $ONOO^-$ breaks down into a hydroxyl free radical (OH), causing oxidative damage and the generation of more free radicals in a vicious cycle (12). The chain reaction of free radical propagation may account for the time-dependent nature of many diseases (13). In previous studies, the total NO level in the retina was consistently higher in IOP-elevated eyes than in control eyes, which indicates that the retina may be at risk for significant oxidative damage (14,15). Glaucoma neuropathy evidently is a type of free radical stress, and NOS plays an important role in the formation of free radicals. In the present study, we used eNOS (which is a member of the reactive oxygen species family) to study the roles played by free radical gene polymorphisms in primary open angle glaucoma (POAG). Two major gene polymorphisms in the eNOS gene are the eNOS intron-4 a-deletion/b-insertion and promoter −786 T-to-C, which are known to be associated with many other diseases (16). Therefore, we selected these two gene polymorphisms as candidate genes to study their relationship with POAG.

$NO^\bullet$ autoxidizes to nitrite (NO_2^-) and nitrate (NO_3^-) (3,12). NO_2^- is a substrate for myeloperoxidase (MPO), a heme enzyme secreted by activated phagocytes. MPO is an abundant enzyme that is involved in the production of free radicals. Indeed, MPO uses hydrogen peroxide (H_2O_2) and NO_2^- to generate reactive nitrogen species that are reduced by nitrate tyrosyl in vitro (4,12). These reactions may be physiologically relevant because human neutrophils use the MPO-H_2O_2-NO_2^- system to chlorinate and nitrate tyrosine analogues (17). An important functional single nucleotide polymorphism (SNP) was recently identified in the promoter region of the MPO −463 gene, consisting of a G-to-A substitution. Previous studies demonstrated that the G allele (in contrast to the A allele) creates a strong SP1 binding site, which correlates with a 25-fold enhancement of the gene transcription activity (18). For this reason, we also chose the MPO −463 gene polymorphism as one of the candidate gene polymorphisms for this study.

Although investigators have shown intense interest in the role of reactive nitrogen species in host defense mechanisms and oxidative tissue injury, our understanding of their relationship with POAG is still limited. Therefore, we compared the distributions of two major eNOS gene polymorphisms (eNOS intron 4 and eNOS promotor −786) and the MPO −463 G-to-A gene polymorphism between POAG patients and normal controls.

MATERIALS AND METHODS

This was a case control study. We investigated a total of three polymorphisms: eNOS intron −4 a-deletion/b-insertion and b-insertion, eNOS promotor −786 T-to-C, and MPO −463 G-to-A. The prevalence of these polymorphisms was compared between a control group and a group of patients with POAG. An odds ratio was used to calculate the frequencies of alleles.

We enrolled patients with POAG from the Department of Ophthalmology, China Medical University Hospital, from May 2003 to July 2003. All of the patients received serial ophthalmic examinations that included IOP, visual acuity, automated perimetry, gonioscopy, optic disc examination, and retinal examination. Patients with ocular diseases other than POAG were excluded from our study. The volunteers in the control group were examined by the same ophthalmologist. If there was any doubt that a patient had glaucoma, he or she was excluded from the study. The POAG patients included in this study had to meet one of the following criteria from both the visual field and the optic nerve categories:

The visual field criteria included 1) at least two abnormal visual field tests by Humphrey automated perimetry, as defined by computer-based objective criteria; and 2) the presence of one or more absolute defects in the 30° central visual field, attributed to glaucomatous visual field loss.

The optic disc criteria (optic disc damage present in fundus photographs) were 1) a horizontal or vertical cup-to-disc ratio of 0.6 or more, and 2) the narrowest remaining neuroretinal rim was 20% or fewer disc diameters.

According to the ophthalmologic criteria, patients with disc and field changes attributed to causes other than POAG were excluded.

This study was carried out with the approval of the Human Study Committee of the China Medical University Hospital. Informed consent was obtained from all patients who participated in this study. The genomic DNA was prepared from peripheral blood by a Genomaker reagent kit (Blossom, Taiwan).

For this study we enrolled 100 healthy volunteers (50 women and 50 men) and 66 patients with POAG (33 women and 33 men). The patients with POAG ranged in age from 20 to 70 years (mean = 55 years) and were unrelated. The volunteers ranged in age from 52 to 71 years (mean = 50 years), and were free from ophthalmic disease. Furthermore, all of the volunteers were Chinese

and unrelated. All of the patients were followed for 2–8 years (mean = 5 years). Ten of the patients had undergone trabeculectomy, and two of these 10 patients had undergone trabeculectomy twice in different sites. Fifty patients in the POAG group controlled their intraocular pressure with topical drugs. Each patient used an average of 1.3 types of antiglaucomatous drugs. Nine patients did not require drugs to control IOP after trabeculectomy.

We used PCR to identify the genotypes of all of the NO- and MPO-related genes. PCR of the polymorphisms was carried out to a total volume of 50 μl, containing genomic DNA (2-6 *p*mole of each primer), 1 × Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 U of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) The primers for the PCR reaction were 5′-CGGTATAGGCACACAATGGTGAG-3′ (forward primer) and 5′-GCAATGGTT CAAGCGATTCTT-3′ (reverse primer).

For the eNOS intron 4, we loaded 10 μl of the products into 3% agarose gel containing ethidium bromide for electrophoresis, and each allele was recognized according to its size. The eNOS polymorphism at intron 4 was analyzed by PCR amplification followed by restriction analysis by *NgoMIV* (New England Biolabs, Beverly, MA) digestion. The a-deletion/b-insertion and b-insertion alleles showed up as 190-bp and 114-bp, respectively, on agarose electrophoresis. The b-insertion allele was 304-bp (Fig. 1). The region containing the polymorphic site within promoter-786 of the eNOS gene was amplified and then digested by *Taq* I (New England Biolabs). The T allele was 135 bp + 114-bp, and the C allele was 249-bp as shown on electrophoresis (Fig. 2). Molecular analyses of the patients and controls were performed in the same laboratory at the same time, and the gels were inspected by investigators who were blinded to the clinical phenotype of the individuals being studied.

The MPO polymorphism at position -463 was analyzed by PCR amplification followed by restriction analysis by *Ava* I (New England Biolabs, Beverly, MA) digestion. The primers for the PCR reaction were

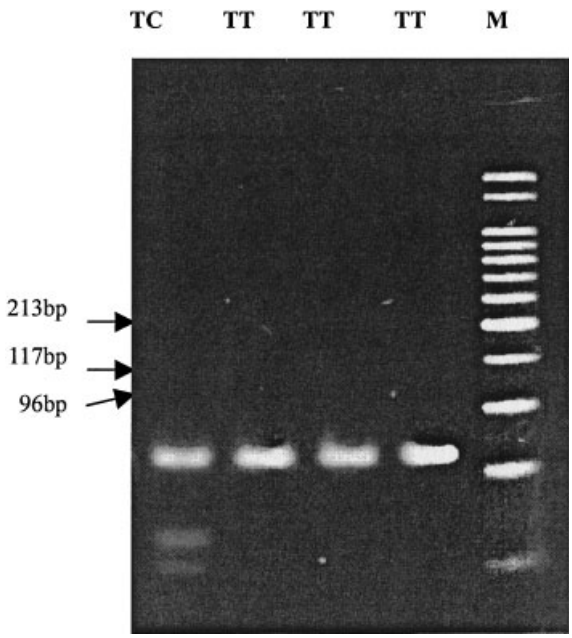

Fig. 2. The eNOS promoter −786 genotype panel shows a 3% agarous gel stain with ethidium bromide after PCR amplification. The T allele was 135 bp + 114 bp, and the C allele was 249-bp as shown on electrophoresis.

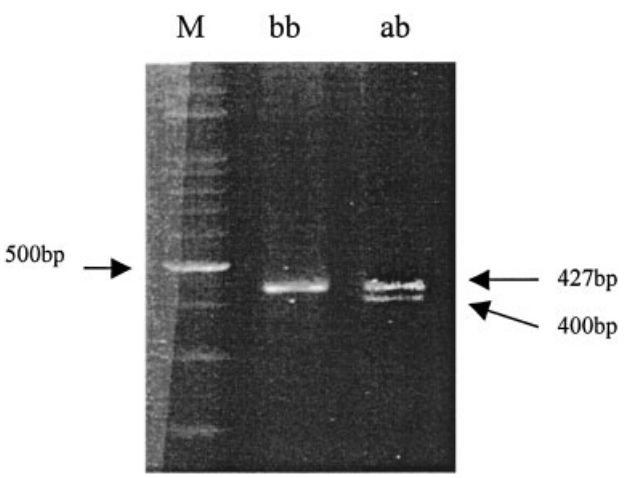

Fig. 1. The eNOS intron 4 genotype panel shows a 3% agarous gel stain with ethidium bromide after PCR amplification. The a-deletion/b-insertion and b-insertion allele showed up as 190-bp and 114-bp on agarose electrophoresis. The b-insertion allele was 304-bp.

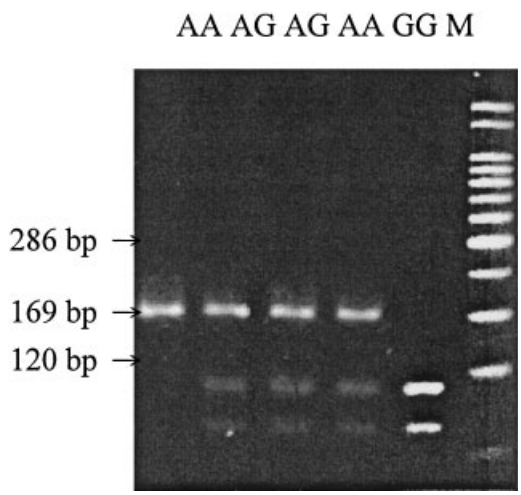

Fig. 3. The MPO polymorphism at position −463 genotype panel shows a 3% agarous gel stain with ethidium bromide after PCR amplification. The G allele was 169 bp + 120 bp, and the A allele was 289-bp as shown on electrophoresis.

5′-CGGTATAGGCACACAATGGTGAG-3′ (forward primer) and 5′-GCAATGGTT CAAGCGATTCTT-3′ (reverse primer) (Fig. 3).

RESULTS

We compared the distribution of the eNOS intron 4 polymorphism between the healthy control and POAG patient groups by means of Fisher's exact test. There was no significant difference between the two groups ($P = 0.481$; Table 1). The distribution of the genotypes in the control group revealed 0 (0%) a-deletion homozygotes, 17 (17.0%) a-deletion and b-insertion heterozygotes, and 83 (83.0%) b-insertion homozygotes. The distribution of the genotypes in the POAG group revealed 0 (0%) a-deletion homozygotes, 10 (15.4%) a-deletion and b-insertion heterozygotes, and 56 (84.6%) b-insertion homozygotes. There was no significant difference in the frequency of each allele (Table 2).

TABLE 1. Distribution of eNOS intron 4 gene polymorphism between the healthy control subjects and POAG patients*

Genotype	Glaucoma, total: 66 (%)	Normal control, total: 100 (%)	Total
a-deletion	0 (0)	0 (0)	
a-deleton/b-insertion	10 (15.4)	17 (17)	
b-insertion	56 (84.6)	83 (83)	
Total	66	100	166

*Fisher' exact test: $P = 0.481$ (> 0.05).

TABLE 2. The allelic frequencies of eNOS intron 4 in healthy subjects and POAG patients*

Allelic frequency	Glaucoma, total: 66 (%)	Normal control, total: 100 (%)	Total
Allele a-deletion	10 (7.7)	17 (8.5)	
Allele b-insertion	122 (92.3)	183 (91.5)	
Total	132	200	332

*Fisher' exact test: $P = 0.483$ (> 0.05).

TABLE 3. Distribution of eNOS promoter −786 gene polymorphism between the healthy control subjects and POAG patients*

Genotype	Glaucoma, total: 66 (%)	Normal control, total: 100 (%)	Total
T/T	55 (83.3)	84 (82.9)	
T/C	11 (16.7)	16 (17.1)	
C/C	0 (0)	0 (0)	
Total	66	100	166

*Fisher' exact test: $P = 0.555$ (> 0.05).

TABLE 4. The allelic frequencies of eNOS promoter −786 in healthy subjects and POAG patients*

Allelic frequency	Glaucoma, total: 66 (%)	Normal control, total: 100 (%)	Total
Allele T	121 (91.7)	184 (91.4)	
Allele C	11 (8.3)	16 (8.6)	
Total	132	200	332

*Fisher' exact test: $P = 0.554$ (> 0.05).

TABLE 5. Distribution of MPO −463 gene polymorphism between the healthy control subjects and POAG patients*

Genotype	Glaucoma, total: 66 (%)	Normal controls, total: 100 (%)	Total
A/A	2 (3.03)	2 (2.00)	
A/G	11 (16.67)	27 (27.00)	
G/G	53 (80.30)	71 (71.00)	
Total	66	100	166

*Fisher' exact test: $P = 0.292$ (> 0.05).

TABLE 6. The allelic frequencies of MPO −463 in healthy subjects and POAG patients*

Allelic frequency	Glaucoma, total: 66 (%)	Normal controls, total: 100 (%)	Total
Allele A	15 (11.36)	31 (15.50)	
Allele G	117 (88.64)	169 (84.50)	
Total	132	200	332

*Fisher' exact test: $P = 0.183$ (> 0.05).

The distribution of the eNOS promoter −786 gene polymorphism in the healthy control and POAG patient groups was significantly different (Fisher's exact test, $P = 0.555$; Table 3). The distribution of the eNOS promoter −786 genotypes in the control group revealed 84 (82.9%) T/T homozygotes, 16 (17.1%) T/C heterozygotes, and 0 (0%) C/C homozygotes. The distribution of genotypes in the POAG group revealed 55 (83.3%) T/T homozygotes, 11 (16.7%) T/C heterozygotes, and 0 (0%) C/C homozygotes. There was no significant difference in the frequency of each allele (Table 4).

There was no marked difference in the distribution of the MPO −463 G-to-A gene polymorphism between the healthy control group and the patient group (Fisher's exact test, $P = 0.183$; Table 6). The distribution of the genotypes of the MPO −463 gene polymorphism in the control group revealed 92 (2.0%) A/A allele homozygotes, 27 (27.0%) A/G allele heterozygotes, and 71 (71.0%) G/G allele homozygotes. There was no significant difference in the MPO −463 genotype

distribution between the two groups (Fischer's exact test, $p = 0.292$; Table 5).

Our results showed that there was no significant difference in the eNOS intron −4 a-deletion/b-insertion, eNOS promoter −786 T-to-C polymorphism, and MPO-463 G-to-A gene polymorphism between the POAG patients and normal controls. Therefore, we conclude that these polymorphisms are not useful markers for POAG in Chinese patients.

DISCUSSION

NO isoenzymes have been identified in all regions of the eye (19). Major sites of outflow resistance (trabecular meshwork and Schlemm's canal), collecting channels, and particularly the ciliary muscle are rich in NOS (7). NO is an important mediator of the homeostatic functions of the eye, including regulation of aqueous humor dynamics, neuronal visual processing, local modulation of ocular blood flow, and control of retinal ganglion cell death by apoptosis (19,20). Information on the NOS system in human eyes is limited. Nathanson and McKee (22) proposed that topical and intracameral application of NO can alter the aqueous humor outflow facility in rabbits and monkeys. A deficit in NOS-like immunoreactivity has been found in the ciliary muscle and outflow pathway in POAG (23,24). These studies revealed that the existence of NO in the anterior segment of the eye can assist the outflow of aqueous humor. Nevertheless, NO levels in the aqueous humor have been found to be higher in glaucoma patients than in cataract patients, and NO levels vary significantly in different types of glaucoma (21,22). This may be because NO will increase in glaucoma patients in compensation for intraocular pressure induced optic nerve damage. However, too much NO will induce oxidative damage to the eye. Chronic overproduction of NO has been reported to be associated with neuronal degeneration, such as in Parkinson's disease and Alzheimer's disease (21,25). Moreover, the retina has very high levels of polyunsaturated fatty acids (PUFA), which are susceptible to oxidative damage. It is believed that NO either attacks the double bonds on the PUFA membrane or modifies genetic nuclear materials (3,19), causing necrotic and apoptotic cell death. The detailed mechanism that controls how retinal NOS activity is regulated under elevated IOP remains unclear. Further studies are required to elucidate the retinal NO regulatory mechanism under elevated IOP in different NOS subgroups. The selective inhibition of NOS caused conflicting results in previous neuronal protection studies (8,26).

In conclusion, small amounts of NO are beneficial to the retina because it upregulates the blood circulation and thus facilitates the flow of metabolites. Excessive NO, however, may damage the retinal tissues by a free radical oxidative mechanism (3,27). The exact roles that NO and MPO genes play in POAG patients is still unclear. Although the polymorphisms of free radical genes investigated in the current study showed no significant differences between the POAG patients and the control group, we plan to investigate the role that other free radical-related genes may play in POAG, as well as the role that "proteinomics" of free radicals may play in retina ganglion cells under glaucoma stress. The potential applications of antioxidants in retinal therapy deserve more attention. There is a debate as to whether a preventive strategy (such as antioxidative supplements) can complement the stringent control of IOP to combat glaucoma-related oxidative stress. Therefore, the NOS inhibitor should be applied with caution in glaucoma patients until the role of NOS is fully characterized.

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