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ORIGINAL ARTICLE

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# The impact of endothelial nitric oxide synthase polymorphisms on long-term renal allograft outcome

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**Abstract** A major manifestation of chronic allograft failure (CAF) is the accelerated onset of atherosclerotic lesions within the graft. Polymorphisms in the endothelial nitric oxide synthase (eNOS) gene have been implicated in the pathogenesis of native atherosclerosis. This study tested the hypothesis that polymorphisms in eNOS are associated with susceptibility to CAF after cadaveric renal transplantation. The patient cohort comprised 140 renal transplant recipients who had received their transplants between 1985 and 1997 at the Oxford Transplant Centre and included 61 patients with biopsy-proven CAF and 79 with stable graft function for at least 10 years (long-term survivors, LTS). Genotyping for one polymorphism in the promoter region and two polymorphisms in the coding regions of the eNOS gene was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP). No association was found between any genetic variant and the development of CAF, even after stratification for other known risk factors. Statistical analysis revealed that all three polymorphisms were closely linked. We conclude that recipient eNOS gene polymorphisms do not alter the risk of CAF after renal transplantation.

**Keywords** eNOS polymorphisms · Long-term transplant outcome

## Introduction

Chronic allograft failure (CAF) is the most common cause of graft failure after renal transplantation [1, 2]. The pathophysiology of CAF involves graft injury by both alloantigen-dependent and independent factors. Clinically, it has been defined by the Alexis Carrel Conference [3] as a slow but variable decline in renal function associated with new or aggravated hypertension and proteinuria and can be confirmed by histological assessment of transplant core biopsies [4].

A prevalent manifestation of CAF is the accelerated development of atherosclerotic lesions within the graft [5]. The histological similarities that exist between native atherosclerosis and post-transplant arteriosclerosis suggest similar risk factors for initiation and progression. Nitric oxide (NO) plays an important role in the physiology of blood vessels, where its production is catalysed by the enzyme endothelial nitric oxide synthase (eNOS). NO appears to inhibit the important early stages of atherosclerosis, in part by down-regulating the expression of adhesion molecules and chemokines that

promote monocyte–endothelial cell interaction [6]. A recent study showed plasma levels of NO to be significantly higher in hypertensive patients with coronary artery disease (CAD) than in normotensive CAD patients and controls. Furthermore, high levels of plasma NO were associated with a polymorphism at position 894 (codon 298) of the eNOS gene [7]. Thus, genetic variation in the eNOS gene may influence the development of atherosclerotic disease.

This hypothesis has been explored in native atherosclerosis, where a number of positive associations between eNOS polymorphisms and cardiovascular diseases has been identified [8, 9, 10, 11]. Polymorphisms studied include two single nucleotide polymorphisms: a G-to-T substitution at position 894 (codon 298) [9] and a T-to-C substitution at position −786 in the gene promoter [12]. A third silent single nucleotide polymorphism at position 774 causes a change at codon 258 in exon 6. This has been shown to be in close linkage with the variant at position −786 [13]. A further two variable-number tandem repeat (VNTR) polymorphisms in intron 4 and intron 13 have been associated with CAD and hypertension, respectively [11, 14]. While the functional significance of these polymorphisms has not been conclusively demonstrated, Wang et al. showed that approximately 30% of the variance in NO levels could be attributed to the 894G-to-T polymorphism [7, 15]. Additional studies have linked this variant to hypertension [8, 16], myocardial infarction [9, 10], coronary spasm [17] and coronary heart disease [10]. The −786T-to-C polymorphism has also previously been associated with significant variation in eNOS promoter activity [11] and has been associated with coronary spasm in a Japanese cohort [17], although these findings were not replicated in an Australian Caucasoid group [18].

Not only is NO likely to be important in mediating the atherosclerotic changes of CAF, but it may also play a role in mediating ischemia–reperfusion injury. Experimental inhibition of NO production is associated with increased production of free radicals after ischemia–reperfusion injury [19], and the augmentation of local NO results in decreased endothelial inflammation [20, 21]. In human studies, increased endothelial expression of eNOS after renal reperfusion is associated with enhanced recovery from renal ischemia and improved graft function [22]. Delayed graft function, a clinical indicator of ischemia–reperfusion injury, is strongly associated with poor long-term graft outcome [23, 24]. Thus, genetic variation in eNOS function may result in diminished availability of NO following ischemia–reperfusion injury, thereby contributing to local oxidative stress and end organ damage and, subsequently, to the development of CAF.

The aim of this study was to determine whether polymorphisms in the recipient eNOS gene are associated with susceptibility to CAF after renal transplantation.

## Patients and methods

### Patients

This retrospective study was approved by the Central Oxford Research Ethics Committee. The patient cohort consisted of 140 Caucasoid renal transplant recipients, all of whom had received their transplants between 1985 and 1997 at the Oxford Transplant Centre. Of these, 61 patients had biopsy-proven chronic allograft failure (CAF group) according to the criteria established by the Alexis Carrel Conference [3]. These patients were compared with 79 long-term survivors (LTS group), defined as patients who had had stable graft function for greater than 10 years. All patients had received similar post-operative care and cyclosporine-based immunosuppression.

### eNOS genotyping

Primer pairs were designed for the eNOS polymorphisms (Genbank accession no. D26607) by polymerase chain reaction with sequence-specific primers (PCR-SSP, Table 1). We determined the unique sequence specificity of each primer, using the database National Centre for Biotechnology Information (NCBI) BLAST sequence analysis (http://www.ncbi.nlm.nih.gov). Primers were manufactured by Cruachem (Paisley, UK) and were optimised for each reaction. Control primers that amplify a non-polymorphic region of HLA-DRB1 were used to verify DNA amplification [25].

Genotyping of each eNOS variant studied was performed according to a previously published PCR-SSP protocol [25]. In brief, DNA was amplified in 13-μl PCR reactions, where the final reaction mixture consisted of 67 mmol Tris-base pH8.8, 16.7 mmol ammonium sulphate, 2 mmol magnesium chloride, 0.01% v/v Tween 20, 200 mmol of each dNTP, between 1and 4 mmol of either allele-specific primer and the consensus primer, 0.1 mmol of DRB1 control primers, between 0.01 and 0.1 μg DNA and 0.1,875 units Taq polymerase.

**Table 1** eNOS genotyping primers

| Genetic variant | Amplicon size | Allele-specific primers | Concentration of primer in mix (μl) | Consensus primer | Concentration of primer in mix (μl) |
|---|---|---|---|---|---|
| 894G | 200 | CTGCAGGCCCCAGATGAG | 0.3 | CTGCAAACCACTCCAGCCT | 0.3 |
| 894T | | GCTGCAGGCCCCAGATGAT | 0.3 | CTGCAAACCACTCCAGCCT | 0.3 |
| 774T | 225 | GGCTACCGGCAGCAGGAT | 0.06 | TGCTGTGGGGAGTGGGCC | 0.06 |
| 774C | | GGCTACCGGCAGCAGGAC | 0.06 | TGCTGTGGGGAGTGGGCC | 0.06 |
| −786T | 188 | CATCAAGCTCTTCCCTGGCT | 1.2 | CCTCCCAGCCCCAATTTCC | 1.2 |
| −786C | | CATCAAGCTCTTCCCTGGCC | 1.2 | CCTCCCAGCCCCAATTTCC | 1.2 |

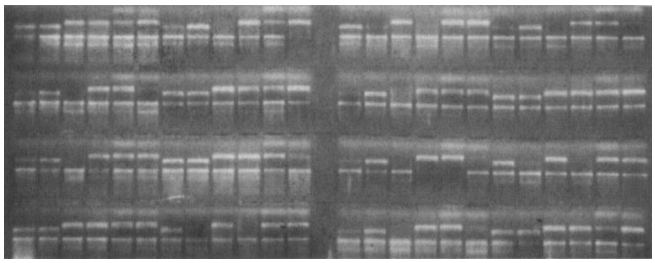

**Fig. 1** An example of a PCR-SSP electrophoretic gel used in this study. The control primer amplicon is present as the lower amplicon in each well. Missing or ambiguous results were repeated

DNA was amplified in PTC-200 (MJ Research) or GeneAmp PCR system 9600 (Perkin–Elmer) thermal cyclers under the following conditions: 1 min at 96 °C, 45 s at 70 °C, 45 s at 72 °C, 21 cycles of 25 s at 96 °C, 50 s at 65 °C and 45 s at 72 °C; four cycles of 24 s at 96 °C, 60 s at 55 °C and 120 s at 72 °C. An aliquot of the PCR reaction plus 10 µl loading dye was loaded into a 1% agarose gel containing ethidium bromide in 0.5× Tris borate EDTA solution. Gels were electrophoresed and were photographed under ultraviolet light (320 nm) with a Polaroid Land camera, as shown in Fig. 1.

### Statistical analysis

Gene frequencies were measured as genotype, allele and phenotype frequencies. Associations between the genotype frequencies were assessed using the $\chi^2$-test (with Yates' correction) and Fisher's exact tests.

## Results

The frequencies of the eNOS polymorphisms in the LTS and CAF groups are presented in Table 2. Gene frequencies were comparable with those previously demonstrated in Caucasoid patients [13] and did not vary from Hardy–Weinberg equilibrium. No significant differences between groups were identified.

This cohort of patients had previously been included in a multivariate analysis of risk factors of CAF in renal transplantation [26]. The two groups were then stratified according to risk factors identified in this and other studies, including HLA matching, cold ischaemia time, delayed graft function, the number and severity of acute rejection episodes and serum cholesterol levels at 1 year post-transplantation. No significant associations were detected in these subgroups (data not shown).

A previous study has shown the promoter polymorphism −786T to be associated with the 774T polymorphism [13]. Analysis of our data showed strong linkage disequilibrium between 774C and 894G ($\chi^2 = 157$). Both polymorphisms were also in linkage disequilibrium with the promoter polymorphism −786T, but the correlations were not as strong ($\chi^2 = 36$, $\chi^2 = 43$, respectively).

## Discussion

Previous studies have suggested that genetic variation in the eNOS gene are associated with the development of atherosclerotic diseases, including CAD, myocardial infarction and hypertension. In view of the histological and pathophysiological similarities between native atherosclerosis and CAF after renal transplantation, and the additional role of eNOS in free-radical scavenging and endothelial inflammation after ischaemic injury, the aim of this study was to explore whether polymorphisms in the eNOS gene contribute to susceptibility to CAF. Analysis of a cohort of individuals with CAF was compared with those with stable long-term graft function.

**Table 2** eNOS genotype (*A*) and allele (*B*)frequencies of long-term renal allograft survivors and individuals with chronic allograft failure

| A | | Genotype frequencies | | | |
|---|---|---|---|---|---|
| Genotype | | Chronic allograft failure ($n = 61$) | | Long-term survivors ($n = 79$) | |
| 774 | TT | 29 | 47.5% | 34 | 43.0% |
| | TC | 21 | 34.4% | 37 | 46.8% |
| | CC | 11 | 18.0% | 8 | 10.1% |
| 894 | GG | 27 | 44.3% | 33 | 41.8% |
| | GT | 24 | 39.3% | 38 | 48.1% |
| | TT | 10 | 16.4% | 8 | 10.1% |
| −786 | CC | 6 | 9.80% | 6 | 7.60% |
| | CT | 30 | 49.2% | 45 | 57.0% |
| | TT | 25 | 41.0% | 28 | 35.4% |
| B | | Allele frequencies | | | |
| Allele | | Chronic allograft failure ($n = 122$) | | Long–term survivors ($n = 158$) | |
| 774 | T | 79 | 64.8% | 105 | 66.5% |
| | C | 43 | 35.2% | 53 | 33.5% |
| 894 | G | 78 | 63.9% | 104 | 65.8% |
| | T | 44 | 36.1% | 54 | 34.2% |
| −786 | C | 42 | 34.4% | 57 | 36.1% |
| | T | 80 | 65.6% | 101 | 63.9% |

This failed to show any relationship between eNOS polymorphisms and the development of CAF.

A number of reasons may explain the lack of association seen in this study compared with previous findings that associate eNOS polymorphisms with native atherosclerosis. Firstly, it is possible that genetic variants in the eNOS gene may not cause functional changes that contribute to the pathogenesis of CAF. While there is strong functional evidence of NO involvement in native atherosclerosis [27], it is possible that NO plays a less pivotal role in CAF and, thus, variations in its transcription and structure are of less importance. Furthermore, as eNOS exerts many of its effects within a tightly regulated local milieu, it would also be interesting to determine whether donor polymorphisms in the eNOS gene have a significant effect on the development of CAF. Alternatively, this study was not exhaustive for eNOS polymorphisms, excluding in particular the well-described VNTR polymorphisms in intron 4 and intron 13. It is possible that these variants, or those that are as yet undiscovered, have more significance than the polymorphisms studied here. Indeed, the importance of the contribution of genetic variation in the eNOS is controversial. Much of the evidence associating these polymorphisms with cardiovascular disease has been reported in the Japanese population [8, 9, 14, 17, 28], and studies in Caucasoid cohorts have been less convincing [29, 30, 31]. Failure to identify an association may be the result of divergent contributions of these variants in different ethnic groups. In addition, although stringent inclusion criteria were used for the selection of the patients in this study, CAF may occur at any time after transplantation. Thus, individuals who survive with good transplant function for more than 10 years remain at risk of CAF, blurring the distinction between the two groups. Finally, the lack of association may reflect fundamental pathophysiological distinctions between native atherosclerosis and post-transplant atherosclerosis.

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