Donor ACE gene polymorphism: a genetic risk factor for accelerated coronary sclerosis following cardiac transplantation

D. A. Cunningham, S. J. Crisp, M. Barbir, F. Lazem, M. J. Dunn and M. H. Yacoub

Division of Cardiothoracic Surgery, National Heart and Lung Institute, Imperial College of Science, Technology and Medicine, London, U.K.; Heart Science Centre, Harefield Hospital, Harefield, Middlesex, U.K.

Aims To investigate the role of angiotensin converting enzyme (ACE) (I/D) gene polymorphism in the development of coronary sclerosis after cardiac transplantation.

Methods and results Eighty cardiac transplant recipients (44 transplant associated coronary artery disease; 36 non-transplant associated coronary artery disease) and their donors were genotyped by polymerase chain reaction. The allele frequencies of the recipients in the transplant associated coronary artery disease and non-transplant associated coronary artery disease groups (I=0·47 and 0·48, D=0·53 and 0·52, respectively) did not differ significantly between the groups. However, there was a negative association between the frequency of the I allele in the donor and the development of transplant associated coronary artery disease. The D allele in the donor population of the non-transplant associated coronary artery disease group had a significantly (P<0·01) lower frequency (0·35) than either the transplant associated coronary artery disease group (0·53) or that of the general population (0·57). Other factors analysed were recipient family history, cholesterol levels, age, sex and body mass index, donor age and acute rejection, of which the significant (P<0·05) factors were acute rejection and sex of the recipient.

Conclusion These results suggest that the ACE genotype of the donor organ may be an additional risk factor for the development of coronary artery disease following cardiac transplantation and that tissue rather than circulating ACE could be implicated in the pathogenesis of this disease.

Key Words: Angiotensin, genes, arteriosclerosis, transplantation.

Introduction Transplant associated coronary artery disease is one of the most serious long-term complications of cardiac transplantation, with up to 40% of patients developing the disease by the 4th postoperative year[1]. The aetiology of transplant associated coronary artery disease is unknown, although both immunological[2] and non-immunological[3,4] factors are thought to be involved, resulting in smooth muscle cell proliferation and the later development of lesions similar, but not identical, to those involved in non-transplant associated atherosclerosis. There is increasing evidence which suggests that the renin angiotensin system may be implicated in the pathogenesis of coronary artery disease. The renin angiotensin system is a regulatory system which functions in the maintenance of salt and volume homeostasis and the regulation of blood pressure[5] and also influences vascular tone and cardiovascular remodelling. Initially recognised in the plasma, acting in an endocrine manner (systemic renin angiotensin system) it is now recognised that local tissue renin angiotensin system are found in the kidney, brain and heart (reviewed by Paul et al.[6]). The renin angiotensin system genes renin, angiotensinogen and angiotensin-converting enzyme are all expressed in cardiac tissue and the specific activation of local cardiac renin angiotensin system has been demonstrated in rats with heart failure, without activation of plasma renin angiotensin system[7]. It has been postulated that effects mediated by local renin angiotensin system may be important in the development of disease. Angiotensin-converting enzyme (ACE) inhibitors have been shown to have an inhibitory effect on smooth muscle proliferation after balloon injury in experimental models[8]. In addition, a reduction in coronary events was observed in patients receiving ACE inhibitors in the SAVE[9] and SOLVD[10] clinical trials.
The renin–angiotensin system may be involved in the pathogenesis of cardiovascular disease through its effect on blood pressure, smooth muscle cell proliferation, myocardial metabolism and remodelling, as well as other mechanisms. These effects can be mediated through circulating or locally produced components of the renin angiotensin system.

Recently, several polymorphic variations in the genes of the renin angiotensin system have been identified. Several studies have shown an association between these and the risk of developing cardiovascular diseases, such as coronary artery disease and hypertension, and cardiovascular events such as myocardial infarction. However, others have failed to show such an association and therefore the role of these polymorphisms remains controversial. The influence of the renin angiotensin system on transplant-associated coronary artery disease has not been investigated. In this study we have used polymerase chain reaction to determine the ACE genotypes of both donors and recipients in a group of patients who developed transplant associated coronary artery disease within 2 years of transplantation and a group of transplant patients who were free of this disease.

Methods

Patients

Eighty consecutive patients who had undergone heart transplantation between March 1989 and July 1991 were studied retrospectively. Patients who died within the first post-transplant year were omitted from the study. Patients were selected on the basis of the availability of angiographic data at 1 and 2 years post-transplantation and on sample availability. They were divided into those with no angiographic evidence of transplant associated coronary artery disease (non-transplant associated coronary artery disease group) at 2 years post-transplantation (36 patients; 26 males, 10 females) and those with evidence of the disease (transplant associated coronary artery disease group) (44 patients; 40 males, 4 females). The original heart disease for those in the non-transplant associated coronary artery disease group were ischaemic heart disease (12), various cardiomyopathies (16), viral and non-viral myocarditis (3), valvular heart disease (2) and congenital heart disease (2). For those patients in the transplant associated coronary artery disease group the original diagnosis was ischaemic heart disease (22), various cardiomyopathies (19), viral myocarditis (1), valvular heart disease (1) and congenital heart disease (1).

All patients were on the immunosuppressive agents cyclosporin A and azathioprine. Methylprednisolone treatment was used, when required, to control episodes of acute rejection. None of these patients were treated with ACE inhibitors post-transplant. Cholesterol (total, HDL and LDL) and triglyceride levels were measured at one year post transplant. (At two years post-transplant many patients were treated with lipid-lowering drugs hence lipid levels at 2 years were not included in the multivariate model).

Diagnosis of transplant associated coronary artery disease

The presence of transplant associated coronary artery disease was diagnosed by angiography at annual assessment and was defined as the presence of at least 25% luminal stenosis of one or more coronary arteries or loss of small intramyocardial branches.

Tissue

We determined the ACE genotype of both donor and explained recipient organs from a total of 80 patients receiving heart transplants, of whom 44 had developed transplant associated coronary artery disease and 36 were disease-free 2 years after transplantation. However, as samples were unavailable for four recipients and three donors within the transplant associated coronary artery disease group and for three recipients and two donors in the non-transplant associated coronary artery disease group, a total of 40 transplant associated coronary artery disease recipients/41 transplant associated coronary artery disease donors and 33 non-transplant associated coronary artery disease recipients/34 non-transplant associated coronary artery disease donors were genotyped.

Samples of recipient and donor myocardium were obtained from tissue taken at the time of transplantation and stored under liquid nitrogen. Recipient tissue was harvested from the explanted heart and obtained from either the ventricles or the atria. Donor tissue was obtained as 'trimmings' from either the atria, the aorta or the pulmonary artery of the donor organ.

DNA extraction

Tissues were snap frozen and stored in liquid nitrogen. DNA was isolated by pulverising in liquid nitrogen to a fine powder, digestion with proteinase K (500 μg . ml⁻¹) in 50 mM Tris pH 8.0, 100 mM EDTA, 200 mM NaCl, 0.1% SDS for 20 h at 37 °C and extraction with an equal volume of phenol. The aqueous phase was further purified by transferring to a Corex tube and centrifugation at 9000 rpm for 20 min. The aqueous supernatant was transferred to a fresh tube and precipitated in 0.3 M sodium acetate and 1 volume of propan-2-ol. The DNA precipitate was spooled out and dissolved in sterile water.

Polymerase chain reaction amplification

Primers used were: sense 5’ CTGGAGACCCACTCCCA TCCTTTCT - 3’, anti-sense 5’ GATGTGGCCATCAC
ATTCGTCAGAT $3^{[23]}$. Fifty nanograms of DNA were amplified with 10 pmols of each primer in 3 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl pH 8·4, 0·1 mg ml$^{-1}$ gelatin, 0·5 mM of each dNTP and 1 unit of Taq DNA polymerase for 30 cycles, with denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min. Polymerase chain reaction products of 480 bp (I allele) and 194 bp (D allele), respectively, were visualized after gel electrophoresis. Homozygotes yielded a single product while heterozygotes yielded both products. To eliminate the possibility of mis-typing $^{[24,25]}$, all DD homozygotes were re-amplified (a) with the above primer pair in the presence of 5% dimethylsulphoxide (DMSO) and (b) in a polymerase chain reaction in which the sense primer, above, was replaced with an insertion specific primer 5'-TGGGACCACAGCGCCCGCCACTAC-3' $^{[18]}$ (i.e. amplifying DNA from II and ID individuals only).

Statistics

Univariate analysis
Allele frequencies were determined by the gene counting method, and Hardy–Weinberg's equilibrium was checked by a chi-square test. The chi-square test was applied to the distribution of the II, ID and DD genotypes in the donor hearts of non-transplant associated coronary artery disease and transplant associated coronary artery disease patient groups as a contingency table. A $P$ value of <0·05 was considered to be significant. Differences in allele proportions were compared using 1-sample (comparing against the European figure) or 2-sample (comparing transplant associated coronary artery disease and non-transplant associated coronary artery disease) tests on proportions. Confidence intervals were derived using standard methods $^{[26]}$. Risk factors for transplant associated coronary artery disease were analysed using the chi-square test and the two-sample t-test (parametric analyses) or the Mann–Whitney test (non-parametric analyses).

Multivariate analysis
Logistic regression, with the group status (transplant associated coronary artery disease or non-transplant associated coronary artery disease) as the outcome variable, was performed using S-Plus statistical software. The regressor variables initially included were those for which there was significant, or nearly significant ($P=0·14$), evidence of a difference between the two groups in the univariate analysis i.e. donor genotype, recipient sex, number of rejection episodes, total cholesterol, LDL cholesterol and presenting disease.

Results

Figure 1 shows examples of individuals homozygous for the deletion polymorphism (DD) — 194 bp fragment (lanes 2 and 6), individuals homozygous for the insertion (II) — 480 bp fragment (lane 5), and heterozygotes (ID) (lanes 1, 3 and 4).

Recipient genotypes
Twenty-seven percent of the recipients were homozygous for the deletion allele, which was similar to that of the Northern European population (27%) $^{[27]}$. Furthermore, no significant differences were seen in the frequencies of the recipient genotypes between those patients in the transplant associated coronary artery disease group and those in the non transplant associated coronary artery disease group (Table 1).

Donor genotypes
Twenty-one percent of the donors were found to be homozygous for the deletion allele, which was less than that of the recipient population (27%). However, this difference was not significant. Table 1 shows the results of the genotype analysis for both patient groups. The percentages of donors who were II, ID and DD were 47%, 35% and 18%, respectively, for non-transplant associated coronary artery disease patients and 17%, 59% and 24% for transplant associated coronary artery disease patients (i.e. significant differences between groups were seen; $P=0·02$). In fact, the frequency of the I allele in the donor population in those patients who did
not develop transplant associated coronary artery disease was also significantly higher (0.65) than that of the transplant associated coronary artery disease group and that of the Northern European population (0.43) (Table 2) (P < 0.05). The distribution of the ACE genotypes in all groups was in Hardy-Weinberg equilibrium.

Table 1  ACE genotype frequencies of recipient and donor hearts

<table>
<thead>
<tr>
<th>ACE genotype (n in brackets)</th>
<th>Recipient TxCAD (n=40)</th>
<th>Recipient non-TxCAD (n=33)</th>
<th>Donor TxCAD (n=41)</th>
<th>Donor non-TxCAD (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>24·4 (10)</td>
<td>24·3 (8)</td>
<td>17·6 (6)</td>
<td>16·1 (7)</td>
</tr>
<tr>
<td>ID</td>
<td>58·5 (24)</td>
<td>54·5 (18)</td>
<td>35·3 (12)</td>
<td>36·8 (13)</td>
</tr>
<tr>
<td>II</td>
<td>17·1 (7)</td>
<td>25 (10)</td>
<td>47·1 (16)</td>
<td>45 (16)</td>
</tr>
</tbody>
</table>

ACE genotype frequencies of recipient and donor hearts for patients in the non-transplant associated coronary artery disease and the transplant associated coronary artery disease groups. Significant differences were seen for the genotype distributions between the donor groups (P < 0.02). TxCAD = transplant associated coronary artery disease.

Table 2  Allele frequencies of recipient and donor tissue

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th>D</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor TxCAD</td>
<td>0·53</td>
<td>0·47</td>
</tr>
<tr>
<td>Donor non-TxCAD</td>
<td>0·35†</td>
<td>0·65‡</td>
</tr>
<tr>
<td>Recipient TxCAD</td>
<td>0·53</td>
<td>0·47</td>
</tr>
<tr>
<td>Recipient non-TxCAD</td>
<td>0·51</td>
<td>0·49</td>
</tr>
<tr>
<td>Northern European population‡</td>
<td>0·57</td>
<td>0·43</td>
</tr>
</tbody>
</table>

Allele frequencies of recipient and donor tissue for patients in the non-transplant associated coronary artery disease and the transplant associated coronary artery disease groups as compared with the Northern European population. The frequency of the I allele and that of the D allele were significantly different in the non-transplant associated coronary artery disease donor hearts, compared with all other groups. *Difference in allele frequency between transplant associated coronary artery disease vs non-transplant associated coronary artery disease, 95% CI 0·027–0·034, P < 0·05; †transplant associated coronary artery disease vs Northern Europeans, 95% CI 0·076–0·30, P < 0·05; ‡Taken from Tiret et al., 1992. TxCAD = transplant associated coronary artery disease.

Other risk factors

As the ACE allele frequencies vary considerably between ethnic groups[28] we determined the ethnic origin of both donors and recipients. The percentage of Northern Europeans in the transplant associated coronary artery disease group was 89% for the donors and 92% for the recipients and for the non-transplant associated coronary artery disease group the percentages were 94% and 97%, respectively.

Possible risk factors for the development of transplant associated coronary artery disease were analysed for both the transplant associated coronary artery disease and non-transplant associated coronary artery disease groups and the results are shown in Table 3. Total cholesterol levels and, in particular, LDL cholesterol levels were elevated in the transplant associated coronary artery disease group relative to the non-transplant associated coronary artery disease group at 1 year post-transplant (P = 0·009 and P = 0·011, respectively). In addition, the number of rejection episodes experienced in the first post-transplant year was significantly higher in the transplant associated coronary artery disease group (P = 0·04) and significantly more patients who developed transplant associated coronary artery disease were male (91% vs 72%; P = 0·03). The subject groups did not differ significantly with regard to family history, HDL cholesterol and triglycerides, donor and recipient age, recipient body mass index and the original presenting disease (Table 3).

Logistic regression with regressor variables donor genotype, recipient sex, number of rejection episodes, total cholesterol, LDL cholesterol and presenting disease suggested that there was little evidence of an effect on the development of transplant associated coronary artery disease associated with the last three variables (i.e. their coefficients were not significantly different from zero). Having omitted these variables, significant associations were seen with respect to the ACE genotype of the donor, a 0/1 variable indicating genotype II, (P = 0·008), the recipient sex (P = 0·015) and the number of rejection episodes (P = 0·02), with the ACE genotype being the most highly significant (Table 3, final column). Reduced chance of transplant associated coronary artery disease was associated with genotype II, being female and having few rejection episodes.

Discussion

This study has shown, for the first time, a relationship between the ACE genotype of cardiac transplant donors (but not recipients) and the development of transplant coronary artery disease.

The development of arteriosclerosis in both the transplant and non-transplant situation is characterized by the neointimal proliferation of smooth muscle cells after the initial endothelial injury and this is a major pathological event in the development of vascular occlusion. The progression of the atherosclerotic lesion would be influenced by higher ACE enzyme levels. Indeed, a higher level of plasma ACE has been shown to be associated with carotid wall thickening in humans[29]. There is some evidence, from experiments in animals, that deregulation of local vascular, and not serum, ACE production may be important in the development of the neointimal lesion. In a balloon-injured rat, vascular ACE activity in the injured aorta was significantly higher than in the uninjured aorta of the control group, while serum and lung ACE levels remained the same[30].

The insertion/deletion polymorphism in the ACE gene was shown by Rigat et al.[31] to be a major contributory factor in regulating serum levels of ACE.
The polymorphism appears to exhibit a co-dominant effect, with subjects homozygous for the deletion allele having the highest level of the enzyme and the lowest levels being found in individuals homozygous for the insertion allele, while heterozygotes display intermediate levels. It is likely that this polymorphism exerts a similar effect on tissue ACE levels. Several studies have shown that the frequency of the DD genotype is significantly increased in patients with a variety of cardiomyopathies[32,33], and an earlier study by Cambien et al.[12] demonstrated that the D allele is associated with an increased risk of myocardial infarction in Northern Europeans. Although no association of this polymorphism with myocardial infarction had been identified in some studies[38], an analysis of 15 published studies[34] supports the initial findings of the European multicentre investigation. Thus, an increase in the frequency of the D allele appears to be associated with a variety of both vascular and muscular heart diseases.

Furthermore, some studies have shown that patients carrying the DD genotype are more likely to develop restenosis after angioplasty[35] or to develop atherosclerosis[36] and thickening of the carotid artery[37] while others have failed to find this association[13,38,39]. Mattu et al.[42] found that the DD genotype conferred an increased risk for the development of coronary artery disease in subjects previously unidentifiable by classic risk factors. Thus, an estimate of the actual risk conferred by I/D genotype relative to other risk factors is important.

Multivariate analysis of the ACE genotype and other risk factors for transplant associated coronary artery disease identified the ACE genotype of the donor as a highly significant factor (P=0.008). However, in our transplant recipient population, no differences were seen in the distribution of the genotypes between those who developed transplant associated coronary artery disease within 2 years of transplantation and the disease-free controls. This may reflect the fact that the recipient genotype influences systemic levels of ACE, whereas the localized levels of ACE within the coronary vasculature, where the disease develops, may depend largely upon the genotype of the donor organ. Indeed, transplant coronary artery disease is limited to donor tissue only, with the venous structures up to, but not beyond, the suture point being affected[40]. However, the distribution of ACE genotypes of the donors differed significantly between the non-transplant associated coronary artery disease and transplant associated coronary artery disease groups with a greater proportion of the patients in the non-transplant associated coronary artery disease and transplant associated coronary artery disease group receiving a heart from a donor homozygous for the insertion allele. Indeed, the frequency of the I allele in the non-transplant associated coronary artery disease population was greater than that of both the transplant associated coronary artery disease group and the general population. These results suggest that the I allele may protect the transplant patient against the development of this disease, possibly due to a lower tissue ACE level in these individuals. ACE produces the effector peptide, angiotensin II, which stimulates the growth of smooth muscle cells[41], activating protein kinase C and growth-related genes such as c-fos[42], suggesting a possible mechanism for the observed ACE genotype effect.

The effect of ACE inhibitors on the development of human transplant associated coronary artery disease has not yet been documented but treatment with cilazapril decreased the degree of luminal occlusion in graft vessels from cyclosporin treated animals in a rat model of heterotopic cardiac transplantation[43].

In our study other significant factors identified by multivariate analysis were the number of acute rejection episodes experienced by the transplant associated coronary artery disease patients within the first post-transplant year (P=0.02) and the recipient sex (P=0.015). An association of transplant associated coronary artery disease with acute rejection has previously

---

Table 3 Comparison of characteristics of transplant associated coronary artery disease patients vs non-transplant associated coronary artery disease patients

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>TxCAD (n=44)</th>
<th>Non-TxCAD (n=36)</th>
<th>P (Univariate)</th>
<th>P (Multivariate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family history (%)</td>
<td>41</td>
<td>47</td>
<td>0.64</td>
<td>—</td>
</tr>
<tr>
<td>Total cholesterol (mmol. l⁻¹)</td>
<td>6.92 (1.4)</td>
<td>6.19 (1.6)</td>
<td>0.009</td>
<td>ns</td>
</tr>
<tr>
<td>HDL cholesterol (mmol. l⁻¹)</td>
<td>1.21 (0.3)</td>
<td>1.3 (0.45)</td>
<td>0.36</td>
<td>—</td>
</tr>
<tr>
<td>LDL cholesterol (mmol. l⁻¹)</td>
<td>4.68 (1.04)</td>
<td>3.99 (1.34)</td>
<td>0.011</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mmol. l⁻¹)</td>
<td>2.26 (1.1)</td>
<td>2.1 (1.5)</td>
<td>0.43</td>
<td>—</td>
</tr>
<tr>
<td>Donor age</td>
<td>31 (11)</td>
<td>28 (10)</td>
<td>0.23</td>
<td>—</td>
</tr>
<tr>
<td>Recipient age</td>
<td>48 (12)</td>
<td>45 (12)</td>
<td>0.28</td>
<td>—</td>
</tr>
<tr>
<td>Body mass index, kg. m⁻²</td>
<td>25.88 (7.0)</td>
<td>22.8 (5.76)</td>
<td>0.09</td>
<td>—</td>
</tr>
<tr>
<td>Presenting disease (% IHD)</td>
<td>51</td>
<td>34</td>
<td>0.14</td>
<td>ns</td>
</tr>
<tr>
<td>Rejection episodes</td>
<td>3.89 (2.33)</td>
<td>2.76 (2.76)</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Recipient sex (% male)</td>
<td>91</td>
<td>72</td>
<td>0.03</td>
<td>0.015</td>
</tr>
<tr>
<td>Donor ACE genotype (% DD, ID, II)</td>
<td>24, 59, 17</td>
<td>18, 35, 47</td>
<td>0.02</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Results are expressed as mean or percentage (standard deviation in brackets). TxCAD = transplant associated coronary artery disease.
been demonstrated[1], although other centres have failed to find such a correlation[44-46]. In a larger study conducted at our centre the association with acute rejection was found to be true for early development of the disease only (<2 years post-transplantation), with no correlation with mismatching at the HLA A, B or DR loci[47].

Furthermore, autopsies or explants from patients with severe transplant associated coronary artery disease have shown a lymphocytic endothelialitis, absent in non-transplant associated coronary artery disease arteries[48], suggesting a role for cellular rejection in transplant associated coronary artery disease development. The increase in the incidence of transplant associated coronary artery disease associated with increasing number of rejection episodes demonstrated in this study is less significant than the ACE genotype of the donor but, nevertheless, may contribute to disease development. The influence of recipient sex on transplant associated coronary artery disease is likely to be due to the protective role of female sex hormones, which has previously been documented in the rabbit cardiac allograft[49].

Thus, our results suggest that the donor heart genotype may play an important role in the pathogenesis of transplant associated coronary artery disease and that tissue ACE is more important in atherosclerosis. The use of ACE inhibitors as a prophylactic measure against the development of transplant associated coronary artery disease, particularly in patients who do not have the DD genotype, needs to be investigated further.

S.J.C. received a British Heart Foundation PhD Studentship. We are grateful to Dr A. Mitchell for their help and advice with the statistical analysis.

References

[34] Akagi H, Kim DK, Krueger JE, Wang DS, Dzau VJ, Pratt RE. Inhibition of angiotensin converting enzyme in the


