Coronary microvascular endothelial dysfunction in transplanted children

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Aims To assess the response of the coronary microcirculation to acetylcholine (endothelium-dependent vasodilator) and of adenosine (endothelium-independent vasodilator) in children after heart transplantation and to verify whether endothelial dysfunction is time-dependent.

Methods and Results We studied the endothelial function of 26 asymptomatic children previously submitted to heart transplantation, with normal transplanted hearts and epicardial coronary arteries. Ten untransplanted children served as controls. The response of coronary blood flow velocity to intracoronary infusion of acetylcholine (1·8 μg.min⁻¹) and adenosine (270 μg.min⁻¹) was assessed using a Doppler wire positioned in an epicardial coronary branch. In the study group, coronary blood flow velocity increased slightly during acetylcholine infusion (peak/baseline ratio=1·17±0·22). The ratio was inversely correlated with the length of follow-up (r=−0·50; P=0·0078). The peak/baseline ratio in control children was 1·76±0·73 (P<0·0002 vs study group). After adenosine infusion, the coronary blood flow velocity peak/baseline ratio was 3·75±1·54 in transplanted children and 3·72±1·34 in controls (P=ns).

Conclusions Endothelial dysfunction in paediatric transplanted patients becomes more evident in patients with longer follow-up. This finding could prove useful in the prevention of accelerated arteriosclerosis.

Key Words: Heart transplantation, paediatric, coronary microcirculation, endothelial dysfunction, accelerated graft arteriosclerosis.

Introduction

Accelerated coronary arteriosclerosis limits survival in both adults and children undergoing heart transplantation[1,2] and seems to be related mostly to the interplay of non-immune and immune processes, which initially affect endothelial function[3–5].

Treasure et al.[6] found a progressive reduction of the coronary microvascular response to acetylcholine over the first 3 years after heart transplantation in adults. The time-course of the response of coronary microcirculation to acetylcholine in children, however, is still poorly known. The aim of this study was to assess the response of the coronary microcirculation to acetylcholine, an endothelium-dependent vasodilator, and of adenosine, an endothelium-independent vasodilator, in children studied 1 to 8·2 years after heart transplantation, and to verify if the time-dependence of endothelial dysfunction described in adult patients can also be recorded in transplanted children.

Materials and Methods

Patients

We studied 26 children (mean age 10·9±5·0 years; mean weight 36·3±16·9 kg) who had undergone heart transplantation from April 1986 to October 1995, alive at least 1 year after the operation and who fulfilled the following inclusion criteria: (1) left ventricular mass/volume ratio <1·3 with ejection fraction >0·50[7]; (2) lack of epicardial coronary stenosis >50%; (3) absence of active rejection (multifocal lymphocytic infiltration and associated myocytes necrosis) requiring specific antirejection therapy[8]. The mean follow-up period was
Table 1  Clinical characteristics of study group patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>10.9 ± 5.0 years</td>
</tr>
<tr>
<td>Weight (mean ± SD)</td>
<td>36.3 ± 16.9 kg</td>
</tr>
<tr>
<td>Males/females</td>
<td>18/8</td>
</tr>
<tr>
<td>Follow-up (median; range)</td>
<td>3 years; 1-8.2 years</td>
</tr>
<tr>
<td>Acute rejection/patient (median; range)</td>
<td>2; 0-6</td>
</tr>
<tr>
<td>Cyclosporine levels (mean ± SD)</td>
<td>235.9 ± 62.9 mg·dl⁻¹</td>
</tr>
<tr>
<td>Cholesterol levels (mean ± SD)</td>
<td>167.0 ± 32.5 mg·dl⁻¹</td>
</tr>
<tr>
<td>History of hypertension (Y/N)</td>
<td>2/24</td>
</tr>
<tr>
<td>Ejection fraction (mean ± SD)</td>
<td>0.65 ± 0.09</td>
</tr>
<tr>
<td>Mass/volume ratio (mean ± SD)</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

SD=Standard deviation.

3.5±2.2 years (median 3 years; range 1.0 to 8.2 years). Seven patients were studied twice during the follow-up period, and hence we could include data from 33 procedures performed on 26 patients. Ten additional untransplanted patients (mean age 8.5±4.3 years, mean weight 32.7±16.6 kg), with patent ductus arteriosus with QP/QS <1.5, normal ejection fraction, submitted to catheterization for positioning of a patent ductus arteriosus occluding device, served as controls. The study in the control group was conducted approximately 1 h after the successful positioning of the occluding device, under normal haemodynamic conditions.

Informed consent was obtained for all patients from their parents. The protocol of this prospective study was approved by the Scientific and Ethical Committee of the Bambino Gesù Children Hospital. Clinical and laboratory data are summarized in Table 1.

Eight patients in the study group and all patients in the control group were submitted to a second acetylcholine intracoronary infusion after suspension of halothane administration and disappearance of the anaesthetic agent from expired gas.

Donor heart preservation

The heart was harvested in the usual manner. Cold (4°C), antegrade St. Thomas’s Hospital cardioplegic solution was used for cardiac arrest. Preservation during transport was achieved by storage in a 4°C saline solution (EuroCollins). Ischaemic time has been approximately 90 and 300 min.

Immunosuppressive protocol

All transplanted patients were submitted to the following protocol:

1) Peri-operative immunomodulation: (a) 4 h before operation: cyclosporine A 5–10 mg·kg⁻¹ by mouth (depending on age and renal function) and azathioprine 2 mg·kg⁻¹ by mouth; (b) after the operation: prophylactic polyclonal antithymocyte immunoglobulins (post-operative day 1 to day 3–5) and methylprednisolone 30 mg·kg⁻¹ day⁻¹ (operative day and post-operative day 1, then gradually discontinued).

2) Post-operative immunosuppression: (a) cyclosporine A 10 mg·kg⁻¹ day⁻¹ by mouth as initial dose, followed by a maintenance dose able to achieve a serum concentration of 250–350 ng·ml⁻¹ in the first 3 months, and 200–300 ng·ml⁻¹ thereafter; (b) azathioprine 2 mg·kg⁻¹ day⁻¹, with dose adjustment for white blood cell count >4000·ml⁻¹; prednisone 2 mg·kg⁻¹ day⁻¹ for the first 2–3 weeks.

3) Acute rejection: grade 3A: methylprednisolone 20–30 mg·kg⁻¹ for 3 days; grade 3B: antithymocyte immunoglobulins for 5–7 days; grade 1 and 2 acute rejection is evaluated case by case. Patients who suffer from recurrent rejection are usually treated with the adjunct of chronic methotrexate and/or prednisone administration.

Study protocol

In patients on ACE inhibitors or calcium channel blockers these drugs were discontinued 24–48 h before the study. All patients were anaesthetized, intubated and mechanically ventilated immediately before cardiac catheterization, as routinely performed in our department, in order to maintain stable arterial blood gas values and haemodynamic conditions throughout the study. Fluothane 0.5–0.8%, nitrous oxide 50% in oxygen, intravenous fentanyl 5 mg·kg⁻¹ and 0.1 mg·kg⁻¹ of intravenous pancuronium were used for induction of anaesthesia. Anaesthesia was maintained with halothane 0.5–0.8% throughout the catheterization study. All patients received an intravenous bolus injection of heparin (100 IU·kg⁻¹). An intravenous infusion of nitroglycerin was also started at a dose ranging from 2 to 3 μg·kg⁻¹ min⁻¹ (resulting in a reduction of mean arterial blood pressure of 5–8 mmHg), to prevent coronary spasm and achieve maximal dilatation of large epicardial coronary arteries. Immediately after diagnostic catheterization, endomyocardial biopsy and coronary angiography, a 0.018 inch Doppler guide wire, with a 12 MHz piezoelectric transducer (FlowWire, Cardiometrics Inc, U.S.A.) mounted on the tip was advanced into the proximal third of the left anterior descending (31 cases) or of the circumflex (two cases) coronary artery. In these last two patients the Doppler wire was positioned in the circumflex artery because of a better flow velocity signal. Coronary blood flow velocity was measured by connecting the flow wire to a pulsed Doppler velocimeter (FlowMap, Cardiometrics Inc, U.S.A.). After obtaining a stable baseline flow velocity signal, first acetylcholine (1.8 μg·min⁻¹) and then adenosine (270 μg·min⁻¹) were infused into the left coronary ostium for a period of 3 min each; the infusion rate was 1 ml·min⁻¹ for all substances injected; acetylcholine and adenosine were infused at a concentration of 10⁻⁵ mol·l⁻¹ and 10⁻³ mol·l⁻¹, respectively. A wash-out period of about 5 min was allowed after the end of each infusion. Two electrocardiographic leads (V₅ and D₂₅) in patients...
in whom the Doppler wire was advanced into the left anterior descending coronary artery and V₅ and D₁ in patients in whom the Doppler wire was advanced into the circumflex coronary artery) were continuously monitored throughout the study. Arterial blood pressure was measured invasively through the radial artery. These parameters were continuously monitored and recorded on a magnetic tape recorder and played back on paper at the end of each study.

Data analysis

Data are expressed as mean ± 1 standard deviation. Coronary blood flow velocity is expressed as average peak velocity. Flow velocities and haemodynamic parameters during baseline and following acetylcholine or adenosine administration were compared using the ANOVA for repeated measures. The difference between baseline and peak coronary blood flow velocity after each drug infusion was tested by means of an ANOVA for repeated measures. The peak-to-baseline coronary blood flow velocity ratio was calculated and the comparison between study and control group was performed by a Student’s t-test for unpaired data. The correlation between peak/baseline coronary blood flow velocity ratio after acetylcholine infusion (dependent variable) and a group of independent variables (age, sex, weight, follow-up duration, acute rejection/patient, cyclosporine levels, cholesterol levels, history of hypertension, ejection fraction, mass/volume ratio) was performed using a linear regression model (continuous variables) or a t-test for unpaired data (dichotomous variables), as appropriate. A P value <0.05 was considered statistically significant.

Results

Acetylcholine infusion

Study group

The values of heart rate and mean arterial blood pressure during baseline were similar to those observed following acetylcholine infusion.

Baseline and peak coronary blood flow velocity were 18.0 ± 11.1 cm s⁻¹ and 20.8 ± 11.5 cm s⁻¹, respectively, with a peak/baseline ratio of 1.17 ± 0.22 (Fig. 1). Although very small, the difference between peak and baseline was statistically significant (P=0.0002).

A mild inverse correlation (r=−0.50; P=0.0038) was detected between peak-to-baseline coronary blood flow velocity ratio after acetylcholine infusion and length of follow-up (Fig. 2). No other variable showed any significant association with acetylcholine peak-to-baseline coronary blood flow velocity ratio (Table 2).

Control group

No variation in heart rate and blood pressure was detected. Baseline and peak coronary blood flow velocity were 20.4 ± 7.8 cm s⁻¹ and 35.6 ± 20.6 cm s⁻¹ (P=0.02), respectively. The peak/baseline coronary
In our series, two patients, submitted to a higher concentration (10⁻⁴ M) of acetylcholine infusion, developed transient complete atrioventricular block (one case) and severe sinus bradycardia (one case), requiring suspension of the study.

To our knowledge, this is the first report on the coronary microcirculatory function after paediatric heart transplantation. All patients in the study group showed a markedly depressed response to intracoronary infusion of acetylcholine. Although the difference between peak and baseline coronary blood flow velocity is very small, the behaviour of the different patients is highly consistent, with comparable standard deviations, and this accounts for the statistical significance (P=0.0002). Nonetheless, the increase in coronary blood flow velocity is negligible from the clinical standpoint. In contrast, the control population showed a fairly preserved vasodilatation after acetylcholine infusion, far higher than that recorded in the study group (peak-to-baseline coronary blood flow velocity ratio 1.76 vs 1.17, P=0.0002). The coronary blood flow velocity ratio obtained in our control group is lower than that reported for adults. The acetylcholine concentrations we report were not comparable with those reported in similar studies[9], and it is possible that paediatric patients require higher doses to obtain the maximal response of the coronary microcirculation, like those infused in adults. Nevertheless, the difference in the coronary blood flow velocity response to acetylcholine infusion between the study and control group is striking and confirms the presence of a different behaviour in the coronary microcirculation. It must be noted that, early in our series, two patients, submitted to a higher concentration (10⁻⁴ M) of acetylcholine infusion, developed transient complete atrioventricular block (one case) and severe sinus bradycardia (one case), requiring suspension of the study.

Our data are consistent with previous observations[11-16] in adults, confirming that endothelial dysfunction, in terms of reduced responsiveness to acetylcholine infusion into an epicardial coronary segment, develops early after heart transplantation. Treasure et al.[19], found a similar reduced coronary microvascular response to acetylcholine in adult transplanted patients. They also recorded a progressive decrease in the response, with a mean increase of coronary blood flow of 200% 1 year after transplantation, although the correlation is weak (r=-0.50; P=0.0038).
after heart transplantation and 100% 3 years after heart transplantation using a dose of acetylcholine achieving an estimated final blood concentration of \(10^{-6}\) mol. l\(^{-1}\), probably slightly higher than that achieved in our study. It is of interest that they observed an inverse correlation between patient age and coronary microvascular response to acetylcholine\(^6\), hence, the dramatically reduced response to acetylcholine in transplanted children appears to confirm this previous observation.

However, the response to adenosine, an endothelium-independent vasodilator, is preserved and time-independent and this behaviour is comparable to that observed in the control group. Similar findings have been reported for adult transplanted patients\(^6\).

The way in which this coronary endothelial dysfunction of transplanted children might influence the development of coronary arteriosclerosis remains unknown. Nevertheless, such a consistent finding cannot be underestimated, given the importance of the endothelium in vascular tone regulation. Nitric oxide release is stimulated by many agents, including acetylcholine, ADP, ATP, histamine, bradykinine, substance P, thrombin and calcitonin gene-related peptide\(^10\).

Evidence of humoral rejection importance in the development of graft coronary arteriosclerosis has been reported\(^11\), based on findings of a positive correlation between the presence of anti-endothelial cell antibodies and humoral graft rejection. Patients with persistent anti-endothelial cell antibody positivity showed a significantly higher incidence of graft arteriosclerosis compared with anti-endothelial cell antibody negative controls. A similar correlation between anti-endothelial cell antibodies and graft coronary arteriosclerosis has been described by Dunn and co-workers\(^12\). In contrast, Hosenpud et al.\(^13\) failed to show any relationship between humoral rejection and graft arteriosclerosis, suggesting that a cell-mediated immune response against the endothelium is probably the main cause of graft vascular disease. Our study did not address the immunological mechanism underlying endothelial dysfunction; nonetheless, in our series, the number of acute rejections did not correlate with the development of the impaired response to acetylcholine infusion. Since the great majority of acute rejections are cell-mediated, we may speculate that the main cause of endothelial dysfunction could be a repeated humoral rejection against endothelial cells, which may contribute over time to the development of graft arteriosclerosis. Graft endothelium constitutes the actual barrier between recipient and donor, and it is reasonable that a chronic, repeated injury, whether antibody- or cell-mediated, leads to endothelial function impairment. Endothelial dysfunction is ubiquitous and somewhat time-dependent, and could be one of the mechanisms involved in graft arteriosclerosis, at least in a proportion of patients.

Our patients usually received a double-drug, steroid-sparing immunosuppressive therapy. Only four children were receiving chronic steroids for recurrent rejection; in this subset, the coronary responsiveness to acetylcholine did not differ from that of patients on double-drug immunosuppression therapy. At the moment, we do not know whether the patients receiving steroids will have a higher incidence of coronary arteriosclerosis in the future, when compared with the children on double-drug therapy.

A recent multicentred study on induction immunotherapy in transplanted children\(^14\) reported that patients treated with polyclonal antithymocyte preparations have a significantly lower early mortality, compared with non-treated children. Late mortality due to coronary graft arteriosclerosis (three cases) occurred only in non-treated children, although the difference in terms of overall late mortality failed to reach statistical significance. All of our patients received induction immunotherapy with polyclonal antithymocyte preparations. Nonetheless, they all developed endothelial dysfunction. How induction therapy may influence coronary arteriosclerosis is still unclear.

Denervation clearly plays a role in provoking endothelial dysfunction\(^15-17\), although the overall mechanism remains somewhat uncertain. In general, it contributes to worsening myocardial perfusion by preventing the appropriate vasodilating response of the endothelium to adrenergic stimuli. This action might be synergistic with immuno-mediated endothelial damage. While the immune response varies widely case by case, denervation is similar in all patients: therefore, we believe that it could not be the only determinant factor in the development of graft arteriosclerosis.

**Study limitations**

Coronary blood flow velocity was measured during continuous systemic infusion of nitroglycerin. Nitroglycerin was administered to prevent coronary spasm and to achieve maximal dilation of the large epicardial coronary arteries to maximize the accuracy of coronary blood flow velocity measurements as an index of coronary blood flow. Also, it avoided the need for repeated contrast injections to measure coronary diameters. The initial coronary artery dilation, caused by nitroglycerin may have led to underestimation of the effects of the other vasodilators, and in particular of acetylcholine.

However, Macho and Vatner\(^18\) showed that the reduction of coronary resistance by comparable doses of nitroglycerin was less than 10%, while the reduction given by acetylcholine in normal subjects was greater than 200%. Clearly, the lack of coronary microvascular response to acetylcholine observed in our patients was dramatically abnormal despite the potentially confounding effect of nitroglycerin.

An important difference between this study and all other reports is that our children were always anaesthetized and mechanically ventilated. This is routine policy in our catheterization laboratory, because of the length of the studies and the need for maintaining stable haemodynamic conditions throughout the procedure.
We know that halothane does affect vasodilator response of isolated vessels to acetylcholine \([19]\), which could explain the moderately reduced response to acetylcholine in the control group compared with the coronary flow increments reported in the literature \([20]\). However, halothane might have enhanced the unresponsiveness to acetylcholine in the transplanted patients. We were unable to detect any significant difference in coronary blood flow velocity at baseline and at peak acetylcholine infusion during halothane anaesthesia or after halothane wash-out. Instead, the reduced response recorded in the control group may be linked to the patients’ relatively young age.

A variety of biochemical and cellular phenomena, such as the altered interactions between endothelial cells and leukocytes or platelets, may occur during the early phase after heart transplantation, and these were not investigated \([20–22]\). Moreover, there is experimental evidence that different methods of cardiac preservation and storage, or a prolonged time of ischaemia \([23]\), may also influence the development of early endothelial dysfunction. These aspects were not considered in the present study. Our patients were all studied at least 1 year after heart transplantation. We do not know whether early endothelial dysfunction induced by these factors may lead to sustained impairment of microvessel reactivity to acetylcholine infusion. Whatever the cause, endothelial dysfunction seems to be ubiquitous, while only a relatively small proportion of the transplanted patients develop graft coronary artery disease.

**Conclusions**

Our data compare the results of acetylcholine and adenosine infusion after heart transplantation in children, and suggest that the earlier and lasting damage occurs principally within the endothelium of the coronary microcirculation. Children after cardiac transplantation develop time-dependent endothelial dysfunction. Since endothelial dysfunction seems to occur in all patients while graft atherosclerosis develops only in a subset of transplanted patients, other factors must also play a role in long-term graft function. Therefore, the observed endothelial dysfunction cannot be considered the only cause of accelerated atherosclerosis. Nevertheless, its prevention could potentially prove useful in the late outcome of these patients. Further studies are needed to identify other possible risk factors of coronary atherosclerosis in paediatric cardiac transplanted patients and to test higher doses of acetylcholine in paediatric patients.

**References**


