Impairment of G-protein-mediated signal transduction in the porcine coronary endothelium during rejection after heart transplantation

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Abstract

Background: Endothelial dysfunction is an early event leading to atherosclerosis. It also occurs after orthotopic heart transplantation and can be used to predict the development of intimal hyperplasia in the coronary artery wall. The present study was designed to assess the time course and specific alterations underlying endothelial dysfunction due to rejection after heart transplantation. Methods: A porcine model of heterotopic heart transplantation was used. Preoperative serum typing for the class I antigen of the swine lymphocyte alloantigen was performed to ensure compatibility for this antigen. This permitted survival of the graft with a low grade rejection without immunosuppression. Rings (with or without endothelium) of epicardial coronary arteries of native and transplanted hearts were studied in organ chambers filled with modified Krebs-Ringer bicarbonate solution and compared 1, 30 and 60 days after transplantation. Results: Myocardial contractility was normal in all grafts studied at 60 days after transplantation and all coronary arteries were patent. Myocardial biopsies showed the progression of rejection from day 1 to day 60 after implantation. All endothelium-dependent relaxations were normal one day after transplantation. Endothelium-dependent relaxations to serotonin and to the \textsuperscript{\alpha}-adrenergic agonist UK14304 (which both activate receptors coupled to Gi-proteins) and to sodium fluoride (a direct activator of G-proteins) were decreased 30 days after transplantation, while those to the calcium ionophore, A23187, and bradykinin were shifted to the right and those to ADP were normal. At 60 days, endothelium-dependent relaxations mediated by the Gi-protein pathway were decreased further while the concentration-relaxation curves to the other agonists were further shifted to the right. Endothelium-independent relaxations to the nitric oxide donor, Sin-1, were progressively reduced at 30 and 60 days, but maximal relaxations were maintained at 60 days. Histomorphometric studies showed a progressive increase in the percentage of coronary rings with intimal thickening from day 1 to day 60 after transplantation. Conclusions: The progressive endothelial dysfunction reported in this model of accelerated coronary atherosclerosis after transplantation without immunosuppression involves preferentially the pertussis-toxin-sensitive Gi-protein-mediated pathway. Endothelium-independent relaxations are decreased at 60 days, as are all endothelium-dependent relaxations. Decreased endothelium-dependent vasodilatation may contribute to the development of coronary graft vasculopathy. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Endothelial function; Coronary disease; Signal transduction; Transplantation; Atherosclerosis

1. Introduction

The endothelium plays a key role in the local control of vascular tone and in the maintenance of the homeostasis of the vascular wall through its antithrombotic properties and antiproliferative effects on smooth muscle cells [1–3]. The release of endothelium-derived relaxing factor can be activated by receptors linked to nitric oxide synthase by either pertussis toxin-sensitive (Gi) and insensitive (Gp) G-proteins [4,5]. Endothelial dysfunction is an early event in the development of atherosclerosis and is progressive with time. In the early stages of the atherosclerotic process, the endothelial dysfunction affects the pertussis toxin-sensitive Gi-protein-mediated pathway while the pertussis toxin-insensitive G-protein pathway is intact. At later
stages, the dysfunction becomes generalized and is characterized by a decreased bioavailability of nitric oxide either due to increased degradation or decreased production [6]. Although heart transplantation remains the treatment of choice for medically unresponsive terminal heart disease and is associated with a five-year survival of 70%, coronary graft vasculopathy develops in a majority of transplant recipients and is the main cause of death beyond the first year after transplantation [7,8]. The appearance of accelerated atherosclerosis after heart transplantation is preceded by reduced dilatations of the coronary artery to endothelium-dependent agonists, suggesting the presence of an early endothelial dysfunction. When identified, this is predictive of the development of graft coronary disease one year after graft implantation and of the occurrence of cardiac events and death [9]. The exact mechanisms underlying the coronary endothelial dysfunction after transplantation remain elusive. Clinical evidence has hinted to a selective endothelial dysfunction that occurs after heart transplantation even in patients without overt acute rejection. Patients studied one to five months after graft implantation demonstrate a selective impairment of endothelium-dependent dilatation to acetylcholine, while the dilatation to bradykinin is maintained [10]. Several noxious stimuli may impair endothelial function in the course of transplantation, including exposure to depolarizing cardioplegic solutions [11], preservation solutions, ischemia–reperfusion injury during implantation [12], hyperlipidemia, treatment with cyclosporine A and cytomegalovirus infections. The present study was designed to assess the role of the rejection process on coronary endothelial function after heart transplantation and specifically to evaluate the integrity of the G-protein-mediated transduction pathways in coronary endothelial cells.

2. Material and methods

2.1. Animals and immunologic studies

Fifty large-white swines (EARL de Fresnelles, Boisemont, France) of either sex, aged 10 ± 0.3 weeks and weighing 23 ± 0.8 kg, were used. The experiments were performed in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). All procedures used in this study were approved by the local institutional committee on animal care (Suresnes, France). Preoperative blood samples were drawn for determination of blood type and the class I antigen of the swine lymphocytes alloantigen (SLA) system by the microlymphocytotoxicity technique [13] on swines from the same litter to ensure a rate of recombination between the class I and class II region of the major histocompatibility complex (MHC) of less than 1%. The transplantations were performed between animals compatible for blood type and for the SLA class I antigen. Serum typing was performed at the Institut National de Recherches Agronomiques (INRA, Jouy-en Josas, France).

2.2. Anesthesia

Sedation was achieved with a mixture of tiletamine and zolazepam (15 mg/kg) and atropine sulfate (0.04 mg/kg), injected intramuscularly. Anesthesia was obtained with thiopental sodium given intravenously by continuous perfusion in the donors (8 mg/kg/h) and initial perfusion followed by bolus administration in the recipient swines (15.3 ± 1 mg/kg).

Ventilatory support was established by tracheotomy and intubation in the donor and orotracheal intubation with a cuffed tube in the recipient. Ventilation was maintained with a respirator (Mark 8, Bird Co., Palm Springs, CA, USA), with oxygen supplementation to maintain an arterial oxygen saturation of 95%. Venous access was obtained through the left saphenous vein and the left internal jugular vein for volume replacement with Ringer’s lactate. Venous blood samples were taken for complete hematological and biochemical profiles and for lipid analysis in both donor and recipients. Arterial cannulation was performed through the left internal carotid artery for blood pressure monitoring and arterial gas analysis (ABL300 Radiometer, Tacsel, Neuilly Plaisance, France). The pH was maintained between 7.35 and 7.45 by adjustment of the ventilatory rate and intravenous administration of sodium bicarbonate as needed. A rectal probe was used for monitoring the temperature. Antibiotic prophylaxis was provided with a single intravenous dose of teramycine (10 mg/kg) prior to the incision and intramuscular injection for seven days postoperatively (when applicable).

2.3. Cardioplegia

Donor blood (12 ml/kg) was sampled during the initial phase of anesthesia in a 450-ml bag with 63 ml of citrate dextrose phosphate and it was kept normothermic. Ringer’s lactate (3 ml/kg) was added to the donor blood for a final blood cardioplegia solution composition of 4:1 blood–cristalloid ratio (mean volume of cardioplegia delivered, 268 ± 20 ml). Potassium chloride was then added through a Y-tubing to achieve diastolic arrest (mean, 12 ± 3 mEqiv.) [14].

2.4. Heterotopic heart transplantation

2.4.1. Donor

After median sternotomy and pericardial incision, the heart was prepared by dissection and suture control without ligation of the superior and inferior venae cavae, the right and left superior and inferior pulmonary veins and the left hemi-azygos vein (which enters the coronary sinus
in the swine). After systemic heparinization (3 mg/kg, heparin sodium), the distal right innominate artery was ligated and the proximal portion cannulated with a polyvinyl chloride catheter positioned in the ascending aorta for administration of cardioplegia. After clamping of the aortic arch between the two innominate arteries, asystole was induced through injection of the normothermic blood cardioplegia solution in the ascending aorta at a maximal pressure of 60 mmHg. The heart was vented by incision of the right and left atrial appendages and the blood vessels controlled previously were ligated. Local hypothermia was added by flooding the pericardium with cold physiological salt solution (4°C). After excision, the heart was prepared for implantation by ligation of the atrial incisions and dissection of the adventitial tissue between the ascending aorta and the main pulmonary artery.

2.4.2. Recipient

After a left subcostal transverse incision, the infrarenal abdominal aorta and vena cava were approached retroperitoneally and dissected free of lymphatic tissue. After systemic heparinization (3 mg/kg), the infrarenal aorta was controlled by application of a side clamp. End-to-side anastomosis was performed between the donor’s ascending aorta and recipient’s abdominal aorta using a running suture of 5-0 polypropylene. After clamping of the donor aorta to avoid immediate reperfusion, the side-clamp was released and hemostasis secured. Using the same technique, an end-to-side anastomosis was performed between the donor’s main pulmonary artery and the inferior vena cava (6-0 polypropylene). After reperfusion (ischemic time, 61±3 min), air was removed by needle insertion into the apex and left atrial decompression as needed and normal sinus rhythm was reestablished with direct defibrillation as needed.

2.5. Postoperative care

After standard ventilatory weaning, the animals were left to recover in temperature-controlled quarters and fed standard piglet chow (number 8, Pietrement, Provins, France) and water ad libitum. No immunosuppressive drugs were used. Electrocardiograms were recorded weekly for monitoring of ventricular complex morphology and amplitude. The recipients were sacrificed electively one day (n=5), 30 days (n=9) and 60 days (n=11) after transplantation.

2.6. Explantation protocol and experimental groups

Allograft hearts: Anesthesia, venous access, volume replacement and ventilatory support were established as described above. The abdomen was reentered and complete mobilization of the allograft was performed, taking care not to injure the epicardial surface of the heart. After clamping of the abdominal aorta, the transplanted hearts were arrested with a single intra-arterial injection of potassium chloride (15 mEq/L) and were excised rapidly. Two allografted hearts showed no evidence of rejection (n=2 at 60 days) and were considered separately (non-rejecting group).

Native hearts were excised through a median sternotomy and arrested in the same fashion.

Control hearts: Hearts from normal swines of similar weight and age that received the same tranquillizing drug without transplantation were also used.

Exclusion criteria: Hearts were excluded if the contractility was decreased, as assessed by palpitation at the time of explantation or if the coronary arteries were thrombosed (n=4 at day 30, n=1 at day 60).

2.7. Vascular reactivity

The native, allograft and control hearts were placed in a modified Krebs-bicarbonate solution (composition in mmol/L: NaCl 118.3, KCl 4.7, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, glucose 11.1, CaCl\(_2\) 2.5, NaHCO\(_3\) 25 and calcium ethylenediaminetetraacetic acid 0.026; control solution). Oxygenation was ensured using a 95% O\(_2\)-5% CO\(_2\) gas mixture. The epicardial coronary arteries of the native heart and allograft were dissected free from the epicardium, myocardium and from adventitial tissue and divided into rings (4 mm wide; 20 rings from the allograft and 20 rings from the native heart). Rings from the left anterior descending, left circumflex and right coronary arteries were used randomly but matched between native and allograft preparations in all experiments. In some rings, the endothelium was removed by gently rubbing the luminal surface of the ring with a small wooden applicator.

The vascular reactivity of native and transplanted control coronary arteries was studied in organ chambers connected to an isometric force transducer. Data were collected with data acquisition software (IOS3, Emka Inc., Paris, France). Except when stated otherwise, the studies were performed in the presence of indomethacin (10\(^{-5}\) mol/L; to exclude production of endogenous prostanooids) and propranolol (10\(^{-7}\) mol/L; to prevent the activation of \(\beta\)-adrenergic receptors).

Each preparation was stretched to the optimal point of its active length–tension curve (approximately 4 g), as determined by measuring the contraction to potassium chloride (30 mmol/L) at different levels of stretch and was then allowed to stabilize for 90 min. A maximal contraction was determined with potassium chloride (60 mmol/L). Rings were excluded if they failed to contract to potassium chloride (exclusion rate <5%).

After washing and 30 min of stabilization, endothelium-dependent relaxations were studied in preparations contracted with prostaglandin F\(_2\alpha\) (range, 2·10\(^{-6}\)–10\(^{-5}\) mol/L) to achieve a contraction averaging 50% of the maximal
contraction to KCl (60 mmol/L). Responses to serotonin
\((10^{-10}-10^{-5} \text{ mol/L})\) in the presence of \(10^{-6} \text{ mol/L}\) ketanserin, incubated for 40 min before the addition of serotonin to block serotonin \(5HT_2\) receptors, the calcium ionophore A23187 (\(10^{-5}-10^{-3} \text{ mol/L}\)), sodium fluoride [NaF, 0.5–9.5 mmol/L; in the presence of aluminum chloride (\(10^{-5} \text{ M}\)), added 5 min before the addition of NaF], UK14304, (\(10^{-9}-10^{-5} \text{ mol/L}\), an \(\alpha_2\)-adrenergic agonist), bradykinin (\(10^{-10}-10^{-5} \text{ mol/L}\)), and adenosine diphosphate (ADP, \(10^{-8}-10^{-4} \text{ mol/L}\)) were compared between native, allograft and control coronary rings one, 30 and 60 days after transplantation. Except for potassium chloride, no rings were exposed to more than one agonist in the course of the experiments.

Endothelium-independent responses were studied in rings without endothelium by comparing contractile responses to potassium chloride and relaxations to Sin-1 (\(10^{-8}-10^{-5} \text{ mol/L}\); 3-morpholino sydnonimine, a nitric oxide donor) and the direct smooth muscle relaxant papaverine (\(10^{-7}-10^{-6} \text{ mol/L}\)).

3. Histology

3.1. Endothelial cell coverage

In some animals \((n=4)\) at each time interval, Evan’s blue dye 0.5% (1.5 ml/kg) was injected intravenously 30 min before explantation, for evaluation of the endothelial cell coverage of the coronary arteries. In four pigs from each group, silver nitrate staining was performed for each set of rings at 30 and 60 days after surgery to evaluate the endothelial cell coverage. Coronary artery rings were opened longitudinally and pinned down on a silicone dish under a small amount of tension to ensure a uniform surface. The rings were fixed first for 10 min with buffered paraformaldehyde (4%). They were then washed for 1 min with a HEPES sucrose buffer solution. Silver nitrate (0.25%) was applied for 1 min. Washing was performed for 1 min before a second fixation for 2 min. The rings were exposed to light for 24 h in a cacodylate buffer solution. ‘En face’ endothelial preparations of coronary artery segments were made and examined under the light microscope. The extent of endothelial coverage was estimated by an independent evaluator. Microphotographs of representative areas were taken.

3.2. Myocardium

Surgical transmural myocardial biopsies of all allografts and selected native hearts were taken from the septum, the right and left ventricles of fresh specimens and fixed in formaldehyde (10%). Hematoxylin–eosin–safran staining was performed and the biopsies were evaluated for rejection grade [15], the extent of necrosis and ischemic changes.

3.3. Coronary artery rings

After organ chamber experiments, the coronary rings were fixed in 10% formaldehyde for 20 min at their optimal tension. All formalin-fixed tissue sections were embedded in paraffin and 5 μm sections were stained with orcein. Each section was examined for the presence, extent and distribution of intimal thickening, luminal narrowing, inflammatory infiltrates and disruption of the internal elastic lamina by light microscopy. All histological slides were read in a blinded fashion according to a semiquantitative scale modified from Lurie et al. [16] and published previously [14].

3.4. Electron microscopy

In three animals at 60 days, matched segments of the proximal portion of coronary arteries of native and allografted hearts 60 days after transplantation were harvested for electron microscopy studies. Samples of tissue (1 mm³) were taken from the animals and fixed by immersion in 2.5% glutaraldehyde that was buffered to pH 7.2–7.4 with phosphate buffer for 1 h at room temperature. They were then washed in phosphate buffer and fixed for 1 h in 2% osmium tetroxide in phosphate buffer at 4°C. After dehydration in a graded series of alcohol dilutions, they were embedded in Epon 812 epoxy resin substitute. Survey sections (1 μm) were cut and stained with 1% toluidine blue for examination with the light microscope. Silver/gold ultra-thin sections of representative areas were then cut, mounted on copper grids and stained with uranyl acetate and lead citrate. The ultra-thin sections were examined using a JEOL 100 transmission electron microscope operating at 60–80 kV.

3.5. Drugs

All solutions were prepared daily. Adenosine diphosphate, aluminum chloride, bradykinin, the calcium ionophore A23187, 5-hydroxytryptamine creatinine sulfate (serotonin), indomethacin, ketanserin, papaverine, propranolol, prostaglandin F₂α, sodium fluoride and UK14304 were purchased from Sigma (St-Quentin Falavier, France). Sin-1 was synthesized at the Servier Research Institute.

3.6. Statistical analysis

Relaxations and contractions are expressed as a percentage of the maximal contraction to potassium chloride for each group and expressed as means±standard error of the mean (SEM); \(n\) refers to the number of animals studied. ANOVA studies were performed to compare dose–response curves. A two-way ANOVA (group× concentration) with repeated measures on concentration was done with Greenhouse–Geisser corrections of sphericity (because of repeated measures). The Newman-
Keuls test was used as the post-hoc test. Linear regression with repetition was performed for estimation of EC$_{50}$ values, when possible. Student’s $t$-test for paired/unpaired observations was used for statistical analysis in the comparison of the incidence of intimal hyperplasia at the different time points. Armitage’s linear trend test was used to determine if there was an influence of time on the development of intimal hyperplasia. The Mantel-Haenszel $\chi^2$ test was used for the comparison of the incidence of intimal hyperplasia between native and allograft coronary arteries at the different time points. Differences were considered to be statistically significant when $P$ was $<0.05$.

4. Results

4.1. Vascular reactivity

4.1.1. Endothelium-dependent relaxations in the rejection group

There were no significant differences in the endothelium-dependent relaxations between rings from control and native hearts at all times after transplantation (data not shown). There were no significant differences in relaxations between the native hearts at days one, 30 and 60 (data not shown). There were no significant differences in endothelium-dependent relaxations to all agonists between native and allograft coronary rings one day after transplantation (no evidence of rejection at myocardial biopsy).

Relaxations to serotonin, UK14304 and to sodium fluoride were decreased significantly 30 and 60 days after transplantation (Fig. 1). The relaxation–concentration curves to the calcium ionophore A23187, adenosine diphosphate and bradykinin, were shifted progressively to the right 30 and 60 days after transplantation but there were no statistically significant differences in terms of maximal relaxations (Fig. 2). At 60 days, there were no statistically significant differences between the relaxations to bradykinin in the presence or absence of indomethacin (Fig. 3).

4.1.2. Endothelium-dependent relaxations in non-rejecting animals

In two swines, there was no evidence of rejection in the allografted heart 60 days after transplantation. The endothelium-dependent relaxations to serotonin, sodium fluoride, UK14304 and bradykinin of coronary artery rings from these two animals were compared to the results from allografts showing evidence of rejection 60 days after transplantation ($n=9$). There were statistically significant greater relaxations to all agonists in the non-rejecting hearts compared to rejecting hearts. There were no significant differences between endothelium-dependent relaxations of allograft coronary rings from non-rejecting hearts 60 days after transplantation and either rings from native hearts or allograft coronary rings one day after transplantation for relaxations to serotonin, sodium fluoride and bradykinin. There was a rightward shift of the concentration–response curve to UK14304, but no statistically significant difference in maximal relaxation.

4.1.3. Endothelium-independent relaxations

There were no statistically significant differences in maximal relaxations to Sin-1, one, 30 and 60 days after transplantation (Fig. 4). There was a significant rightward shift of the concentration–response curve to the nitric oxide donor on days 30 and 60 after transplantation. In parallel experiments, there was a significant rightward shift of the relaxation–concentration curve to papaverine (a non
Fig. 3. Cumulative concentration–response curves to bradykinin in rings of porcine coronary arteries with endothelium: native hearts (■), 60 days with indomethacin (○) and without indomethacin (◇) after heterotopic heart transplantation. Responses are given as the percent of relaxation to the contraction induced by prostaglandin F$_{2\alpha}$. Results are presented as means±SEM.

with native rings with endothelium (Table 1). At 30 and 60 days, there was a significant decrease in the amplitude of contraction to potassium chloride of allograft rings without endothelium compared with allograft rings with endothelium (Table 1).

Prostaglandin F$_{2\alpha}$ (range, 2·10$^{-6}$–10$^{-5}$ mol/L) was used to achieve a contraction between 40 and 70% of the maximal contraction to KCl (60 mmol/L). There were no statistically significant differences in the amplitude of contractions to prostaglandin F$_{2\alpha}$ between native and allograft coronary rings with endothelium or between native and allograft coronary rings without endothelium, except at 30 days, when there was a significantly greater amplitude of contraction in native rings with endothelium compared to allograft rings with endothelium (Table 1). A significantly lower concentration of prostaglandin F$_{2\alpha}$ was needed to achieve the target level of contraction (selected to study endothelium-dependent and endothelium-independent relaxations) in both native and allograft rings without

Fig. 4. Cumulative concentration–response curves to Sin-1 in rings of porcine coronary arteries without endothelium: native hearts (■), allografted hearts one day (○), 30 days (▲) and 60 days (●) after heterotopic heart transplantation. Responses are given as the percent of relaxation to the contraction induced by prostaglandin F$_{2\alpha}$. Results are presented as means±SEM. The asterisks indicate statistically significant differences between day 1 and the indicated time for the indicated concentration: * $P<$0.05, ** $P<$0.01, *** $P<$0.001.

NO-mediated direct smooth muscle relaxant) in allograft coronary arteries at 60 days (data not shown).

4.1.4. Contractions

The amplitude of the contraction to potassium chloride was significantly lower in allograft coronary rings compared with the native coronary arteries in both rings with and without endothelium (Table 1). There were no significant differences in the sensitivity to potassium chloride of native and allograft coronary rings at days 1, 30 and 60 after transplantation (Fig. 5). At 30 days, there was a significantly lower amplitude of contraction to potassium chloride in native rings without endothelium compared
Table 1
Contractions to potassium chloride (60 mmol/L) and prostaglandin F$_{2\alpha}$ of native and allograft coronary arteries at different times after heterotopic transplantation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Day 1</th>
<th>Day 30</th>
<th>Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Allograft</td>
<td>Native</td>
</tr>
<tr>
<td>KCl (60 mmol/L) (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with endothelium</td>
<td>10±0.3$^b$</td>
<td>6.5±0.3</td>
<td>10.9±0.3$^b$</td>
</tr>
<tr>
<td>without endothelium</td>
<td>10±1.1$^b$</td>
<td>5.4±1.1</td>
<td>8.9±0.7$^{b,c}$</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$ (% of maximal contraction to 60 mmol/L KCl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with endothelium</td>
<td>60±4</td>
<td>72±6</td>
<td>52±3$^b$</td>
</tr>
<tr>
<td>without endothelium</td>
<td>77±6</td>
<td>91±15</td>
<td>54±9</td>
</tr>
<tr>
<td>Dosage of PGF$_{2\alpha}$ (10$^{-6}$ M) (for achieving target levels of contraction) with endothelium</td>
<td>7.4±0.8</td>
<td>8.3±1.1</td>
<td>7.7±0.7</td>
</tr>
<tr>
<td>without endothelium</td>
<td>2±0$^a$</td>
<td>2±0$^b$</td>
<td>3±0$^a$</td>
</tr>
</tbody>
</table>

*Data is shown as means±SEM; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$.$^a$P<0.05 vs. respective allograft. $^b$P<0.05 vs. native with endothelium. $^c$P<0.05 vs. native. $^d$P<0.05 vs. allograft with endothelium. $^e$P<0.05 vs. native.

endothelium compared to native and allograft without endothelium. At 60 days, a significantly higher concentration of PGF$_{2\alpha}$ was needed to achieve the target level of contraction in allograft rings with endothelium compared to allograft rings without endothelium (Table 1) and compared to native coronary arteries at the same time point.

5. Histology

5.1. Macroscopy

There was an increasing number of peritoneal adhesions from day 1 to day 60 after transplantation but no serious collection or evidence of sepsis. Most hearts showed extensive epicarditis. The coronary rings showed adventitial thickening with prominent neovascularization. Some hearts showed a thin subendocardial fibrosis but none had evidence of transmural necrosis. All coronary arteries studied were patent and there were no intracavitary thrombi in the hearts studied.

5.2. Myocardial biopsies

The myocardium from native hearts was normal. Allografted hearts one day after transplantation showed no evidence of rejection. Grafts at 30 days showed extensive lymphocytic infiltration with an absent-to-moderate amount of necrosis, compatible with International Society of Heart and Lung Transplantation (ISHLT) classification grade III rejection, and limited subendocardial fibrosis, compatible with perioperative ischemic injury [15]. Grafts 60 days after transplantation showed extensive lymphocytic infiltration with prominent fibrosis and a moderate-to-severe amount of necrosis, compatible with the ISHLT classification grade IV rejection.

5.3. Evan’s blue dye staining

There was no staining of blue dye in native coronary artery rings at any time at one, 30 and 60 days and in allografts one and 30 days after transplantation. There was occasional patchy staining of allograft coronary rings 60 days after transplantation, but most grafts were unstained.

5.4. Silver nitrate staining

Coronary rings showed a normal cobblestone pattern of the endothelium in all native hearts. One and 30 days after transplantation, the cobblestone pattern was also present but with less definite borders, a picture compatible with endothelial cell edema (Fig. 6a–b). At 60 days, endothelial cells were present but there were more areas of cells with less defined borders and areas of patchy denudation (Fig. 6c).
5.5. Optical microscopy (semithin slices)

Thin slices of native coronary arteries showed the normal arrangement of the vascular wall with a preserved thin intimal layer (Figs. 7a, 7d). Allograft coronary rings at 60 days showed intimal proliferation and thickening despite preservation of the endothelial cells (Fig. 7b, c and e).

5.6. Electron microscopy

Transmission electron microscopy of native circumflex coronary artery showed normal flat endothelial cells (Fig. 8a). Sixty days after transplantation, extensive irregularity of the endothelial surface with penetration by inflammatory cells was observed. Endothelial cell nuclei were not clearly visible (Fig. 8b).

5.7. Histomorphometric studies of coronary artery rings

There were no pathological changes in the native coronary arteries at days 1, 30 and 60 after transplantation. There were no statistically significant differences in the incidence of intimal cushions between native and allograft coronary artery rings one day after transplantation. There was a statistically significant increase in the incidence of intimal thickening in allograft coronary rings 30 and 60 days after transplantation (Table 2).
6. Discussion

The major finding of the present study is that rejection without major endothelial cell loss causes an alteration of Gi-protein-mediated endothelium-dependent relaxations of coronary arteries early after heart transplantation. Other pathways of endothelium-dependent relaxations and vascular smooth muscle cell function also become affected with an increase in the duration of rejection, in the same manner as in other models of progressive atherosclerosis. Contraction to KCl is decreased in allografts at all times and in the absence of endothelium at 30 and 60 days. The dosage of PGI$_2$ necessary to achieve the target contraction was significantly lower in rings without endothelium.

6.1. Endothelium-dependent relaxations

The present finding demonstrates that the rejection process, without initial ischemia–reperfusion injury and in the absence of the confounding effects of immunosuppressive drugs, causes in itself a coronary endothelial dysfunction without destruction of the endothelial cell lining. This early endothelial impairment specifically involves the pertussis-toxin-sensitive G-protein-dependent pathway. This conclusion is based on the reduced responsiveness to serotonin, the $\alpha_2$-adrenergic agonist UK14304 and NaF and the earlier demonstration that pertussis-toxin-sensitive Gi-proteins mediate the relaxations to these agonists [17,18], which is similar to that observed in chronic

Fig. 7. Representative photomicrographs of optical microscopy (a–e) and electron microscopy of native and allograft coronary arteries 60 days after transplantation. (a) Transverse cut of native left circumflex (LCX) coronary artery ($\times$20). Normal coronary artery with well visible endothelial cell layer. (b) Transverse cut allograft LCX coronary artery 60 days after transplantation ($\times$20). Allograft coronary artery showing a preserved endothelial layer but with subendothelial thickening. (c) Transverse side view of allograft LCX coronary artery 60 days after transplantation ($\times$20) showing extensive intimal proliferation with an irregular surface, compatible with inflammatory changes. (d) Transverse view of native LCX coronary artery ($\times$100) showing a normal endothelial cell layer. (e, next page) Transverse view of allograft LCX coronary artery 60 days after transplantation ($\times$100). The nuclei of endothelial cells are visible but there is a subintimal thickening. (f, next page) Transverse view of native LCX coronary artery ($\times$5000) showing normal flat endothelial cells. (g, next page) Transverse view of allograft LCX coronary artery ($\times$4400) showing extensive irregularity of the endothelial surface with penetration by inflammatory cells. No endothelial cells nuclei are clearly visible.
models of atherosclerosis [17–19]. The alteration in G-protein-mediated relaxations occurs without major loss of endothelial cells morphological changes in the early stages of rejection (at 30 days), most likely due to a functional impairment of the endothelial cells (type I injury) [20]. Later, as the duration of rejection increases, morphological changes occur (type II injury) and contribute to the progression of the endothelial dysfunction [20]. Further evidence for the role of rejection as the cause of the endothelial dysfunction comes from the two animals that were studied two months after transplantation and that had no evidence of rejection in extensive myocardial biopsies and no evidence of endothelial dysfunction. This observation, although interesting, is anecdotal and the number of observations could not be increased because of the difficulty in obtaining isografts in this type of large animal model. This absence of rejection is most likely due to only minimal disparities in the non-MHC antigens, which would explain a lesser rejection reaction [21].

In human clinical transplantation, the period of cardiac preservation and the subsequent reperfusion can cause an initial endothelial dysfunction that has been incriminated in the development of graft coronary vasculopathy [12,22,23]. Ischemia and reperfusion cause a selective decrease in endothelial G-protein-mediated relaxations evoked by aggregating platelets [24–26] and cold ischemia, followed by reperfusion, impairs vasodilatation mediated both by cyclic AMP and cyclic GMP [22,24]. In the present study, there was no evidence of endothelial dysfunction one day after transplantation (24 h of reperfusion), as evidenced by the normal endothelium-dependent relaxations at day 1. This is probably due to the study design in which short ischemic times were chosen rather than clinically relevant preservation times, and to the type of cardioplegic technique used [27]. The absence of significant perioperative endothelial dysfunction thus enabled the study of the effect of the rejection process itself on endothelial control of the tone of the underlying vascular smooth muscle.

Loss of endothelial cells during the preservation period, followed by regeneration, could contribute to the endothelial dysfunction [28,29] since regenerated endothelial cells from porcine coronary arteries show a selective impairment of G-protein-mediated vasodilatation [17] 30
tion at 60 days with only slightly decreased relaxations to the calcium ionophore A23187, adenosine diphosphate and to bradykinin.

The decreased relaxations to sodium fluoride, which directly activates G-proteins and causes, in the porcine coronary artery, endothelium-dependent relaxations that are inhibited by exposure to pertussis-toxin [5], further support the interpretation that the defect in the endothelial cell signaling pathway is at the level of the G-proteins rather than at the level of membrane receptors or of receptor–G-protein coupling. The expression of Gi-proteins in cultured endothelial cells from allograft hearts undergoing rejection is preserved despite the occurrence of reduced endothelium-dependent relaxations mediated by these G-proteins [30]. Based on the study of regenerated endothelial cells after balloon denudation, the impairment of Gi-protein-mediated relaxations may be related to a decreased functionality of the G-proteins, as evidenced by a decreased ADP-ribosylation and GTPase capacity of the regenerated endothelial cells [31].

Other steps of the L-arginine–NO pathway could also be affected in the endothelial dysfunction observed during the rejection process, including a reduced intracellular availability of L-arginine, alterations in signaling mechanisms, modifications of the expression or of the activity of constitutive (type III) nitric oxide synthase or increased degradation of NO [32,33].

The endothelium-dependent relaxations not mediated by Gi-proteins were affected by the rejection process, as shown by the rightward shift in the concentration–response curves to the calcium ionophore A23187, bradykinin and ADP. However, the maximal vasodilator capacity was maintained 60 days after transplantation. Part of this rightward shift may be attributed to the decreased sensitivity of smooth muscle cells to nitric oxide, as indicated by the results obtained with the nitric oxide donor, Sin-1. ADP causes endothelium-dependent relaxations of the porcine coronary artery through binding to P2y receptors that are coupled to Gq-protein but also by increasing cAMP [6]. The rightward shift at 60 days may be due to involvement of any step of this pathway. Bradykinin causes endothelium-dependent relaxations through dual mechanisms: one by binding to bradykinin (BK) membrane receptors, which, when coupled to Gq-protein,
activate the calcium–calmodulin complex leading to activation of nitric oxide synthase and NO production from the endothelial cells. The second pathway involves production of the endothelium-derived hyperpolarizing factor (EDHF), which causes endothelium-dependent relaxation by hyperpolarizing the vascular smooth muscle [34–36]. Thus, the shift of the concentration–response curve to bradykinin could be due to alterations in either pathway. A decreased production of EDHF could also contribute to the decreased efficacy of this agonist, although in some models of atherosclerosis, EDHF compensates for reduced NO-mediated relaxations [37]. The contribution of an increased production of endogenous vasoconstrictor prostanoids to endothelial dysfunction is unlikely from the results obtained with bradykinin in the presence and absence of the cyclooxygenase inhibitor indomethacin.

Native coronary artery rings taken from native hearts showed no decrease in endothelium-dependent relaxations at any time and no morphological changes, although rejection of the heterotopic heart was occurring. This differs from data showing impaired endothelium-mediated vasodilatation in the peripheral vasculature of patients with acute rejection of a pulmonary allograft [38]. This may be due to the slow kinetics of rejection in the present model or may be related either to the species, the type of organ transplanted or to the vascular bed studied. The lack of alteration of coronary endothelium-dependent relaxations in the native hearts is consistent with observations obtained during acute cellular rejection in the same model [14].

6.2. Endothelium-dependent and-independent contractions

The decreased amplitude of contractions to potassium chloride in allograft arteries at all times may be due to an alteration in vascular smooth muscle cells, although rejection could not explain the results found one day after transplantation. Preferential damage to the contractile apparatus of the coronary arteries from the ischemia–reperfusion injury cannot be ruled out. An effect of the surgical procedure on the sensitivity of contractile proteins to calcium is another possibility.

The lower amplitude of contraction to KCl of allografts without endothelium at all times may be due to suppression of an endothelium-derived contracting factor released by the allograft’s endothelium. The lower sensitivity of allograft coronary arteries with endothelium to prostaglandin F₂α, particularly at 60 days, may reflect an alteration of the receptors to prostaglandin, compounded by the damage to smooth muscle cells from the rejection process or to the release of an endothelial factor inhibiting contraction. Induction of nitric oxide synthase by rejection, which could contribute to the decreased contractions, was not found to be operative in acute rejection studies in the same model [14]. The contribution of the endothelial dysfunction to decreased contraction to both potassium chloride and prostaglandin in allograft rings is unclear.

6.3. Endothelium-independent relaxations

The decreased sensitivity of the smooth muscle cells to nitric oxide donor and to papaverine 30 and 60 days after transplantation, as evidenced by the rightward shift in the concentration–response curves, may be due to impairment of the smooth muscle cells as a result of the rejection process or to limitation of the diffusion of NO to the muscle due to intimal hyperplasia and thickening of the arteries observed at the later stages of rejection, which would lessen the amount of NO reaching the smooth muscle cells.

6.4. Rejection and graft vasculopathy

The histological studies performed comparing native and allograft coronary artery rings showed that intimal cushions exist constitutively in coronary arteries of young swines; indeed, they are present both in the coronary rings taken from native hearts and from allografts one day after transplantation, which show no evidence of rejection and have a normal endothelial vasodilator function. These regions represent physiological intimal thickening at the site of flow turbulence and bifurcations [39] and are preferential sites for the subsequent development of atherosclerosis [40]. There was a significant increase in the incidence of intimal thickening of allograft coronary rings as the endothelial dysfunction progressed. Although a cause–effect relationship cannot be established in the present study, a decreased activity of the endothelium-derived nitric oxide by the rejection process could favor proliferation of the smooth muscle cells [41] leading to intimal thickening.

6.5. Clinical implications

Rejection causes progressive endothelial dysfunction and alters the ability of coronary smooth muscle to relax, as described also in chronic models of atherosclerosis, and undermines the key regulatory role of the endothelium in the vascular wall. Low grade subclinical rejection could result in progressive endothelial dysfunction since coronary graft vasculopathy has remained unchecked despite the decrease in the number of treated acute rejection episodes and the introduction of cyclosporine [42]. Nitric oxide induction occurs in the macrophages of various allograft models and may be cytotoxic when produced in large quantities [43]. A state of increased oxidative stress compounded by immunosuppressive drugs could occur after heart transplantation [44], scavenging endothelial nitric oxide [32], lessening its antiatherogenic role and favoring the development of graft vasculopathy.
7. Limitations

The heterotopic non-working heart transplantation model does not reproduce the exact hemodynamic situation of the orthotopic ‘working’ heart. It remains unknown how the hemodynamic changes produced by left ventricular filling in a working heart would affect the endothelial function in the short time frame of this model. Clinically relevant ischemic times and preservation conditions were not duplicated, to minimize the influence of perioperative endothelial and myocardial injury on the subsequent development of endothelial dysfunction and to best isolate the effect of the rejection process itself on vascular reactivity. Longer ischemic times and different preservation techniques may have compounded the alterations demonstrated in the present study. No immunosuppressive drugs were used to minimize potential interactions on endothelial function, especially those of cyclosporine, which has direct toxic effects on endothelial cells [45].

8. Conclusion

Graft coronary vasculopathy is due to chronic rejection of the endothelium. The present study confirms that graft coronary vasculopathy develops in Large-White swine with non-MHC antigen differences within sixty days and is preceded by the appearance of selective endothelial dysfunction that involves G-protein-mediated relaxations, as well as other endothelium-dependent relaxations to a lesser extent. This type of endothelial dysfunction also occurs in other models of atherosclerosis. The present data suggest a role for endothelial dysfunction in the genesis of accelerated coronary atherosclerosis. The impairment of the antiproliferative and antithrombotic properties of endothelium-derived relaxing factors, particularly nitric oxide, could have an initiating or a contributory role in the the migration and proliferation of smooth muscle cells that are early events in the development of atherosclerosis. Other sources of injury to the endothelium in the context of heart transplantation, such as the preservation period, the ischemia–reperfusion injury, the toxic effects of immunosuppressive drugs, postoperative hyperlipidemia and cytomegalovirus infection, may compound the injury caused by rejection and further impair the regulatory function of the endothelium on the vascular wall.

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