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An association between microvascular endothelial dysfunction, transcardiac nitric oxide production and pro-inflammatory cytokines after heart transplantation in humans

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Abstract Endothelial dysfunction anticipates the development of transplant coronary artery disease (TxCAD) observed more than 1 year after transplantation (HTx). We investigated whether in patients early after HTx myocardial inducible and constitutive nitric oxide synthases (iNOS; cNOS) are expressed and cardiac nitric oxide production occurs. Moreover, a possible relationship to alterations in endothelium dependent and independent vasomotor function was assessed. Forty-two transplant recipients were studied 37 ± 5 days after HTx. Microvascular coronary flow velocity reserve (CFVR) was tested endothelium dependent (acetylcholine; 30 µg/min × 5 min/i.c.) and independent (adenosine; 160 µg/min × 5 min/i.c.) by Doppler flow wire. Flow velocity increase by a factor greater than 2 was considered normal. Quantitative coronary angiography was used to assess epicardial vasomotor function in response to the same stimuli. Myocardial iNOS and cNOS gene expression were detected by semiquantitative reversed transcriptase polymerase chain reaction. Plasma nitrite levels (µM) were measured by spectrophotometry. Cytokines (TNF-a, IL-6; pg/ml) were measured by enzyme linked immunosorbent assay. In 26.1 % of patients (n = 11; group A) an impaired endothelium dependent CFVR (1.65 ± 0.23 increase) was observed; in 73.9 % (n = 31, group B) a normal endothelium dependent CFVR (3.0 ± 0.7 increase; P = 0.003) was observed. Myocardial iNOS and cNOS gene expression did not differ between the two groups. Transcardiac cytokine production was noted in 58.8 % of patients for IL-6 and in 53.3 % for TNF-α. Coronary sinus (CS) levels of TNF-α, IL-6 and nitrite were higher in group A. A significant increase in nitrite production was found only in patients with impaired endothelium dependent CFVR (aorta: 43.9 ± 3.7 vs CS: 52.8 ± 5.6, P = 0.05), suggesting transcardiac nitric oxide production. In addition, CS nitrite levels correlated with CS TNF-a levels in patients with impaired CFVR (r = 0.44, P = 0.003). Microvascular endothelium dependent CFVR is impaired in 26 % of patients early after HTx. Activation of cytokines and the NO pathway seem to be involved in this vasomotor dysfunction The association between cardiac nitric oxide production and TNF-a in this group indicates a chronic high immunologic process, which may represent an early and important target for therapy and prevention of TxCAD.

Key words Transplantation · Nitric oxide · Endothelial function · Gene expression · Cytokines

Introduction

The progressive development of transplant coronary artery disease (TxCAD) as a result of chronic rejection is the major limiting factor for long-term survival in patients after cardiac transplantation (HTx). A number of immunologic and non-immunologic factors may contribute independently or in concert to the development of TxCAD [1]. In this regard, Hornick and co-workers recently demonstrated that indirect allorecognition is the predominant immunologic driving force for the progression of chronic rejection; it involves the presentation of donor alloantigens that are shed from the cells of the graft by recipient antigen-presenting cells to recipient T-cells [2]. In addition, conventional risk factors for atherosclerosis such as lipids, hypertension or diabetes may trigger the progression of TxCAD at least in part through alterations of vasoactive mediators [3].

In this regard, it has been shown that endothelial cells may be a predominant cellular target of activation, and damage by the process of chronic rejection and endothelial dysfunction may be identified before macroscopic evidence for TxCAD occurs or myocardial function is impaired [4].

The coronary endothelium plays a key role in protection of the vasculature from injury; it is a target organ in several disease states such as diabetes, hypercholesterolemia, hypertension and in patients with chronic high (smolder) immunologic activity after cardiac transplantation. Endothelial injury leads to the development and progression of TxCAD, myocardial ischemia and infarction [5]. It has been reported that an early endothelial dysfunction after cardiac transplantation precedes and predicts the development of TxCAD [6, 7]. Several vasoactive mediators have been discussed as pathologic key factors leading to TxCAD after cardiac transplantation; these include enhanced endothelial release of the potent vasoconstrictor peptide endothelin-1 (ET) and impaired or increased production of nitric oxide (NO) derived from the constitutive and/or inducible nitric oxide synthases (cNOS; iNOS) [8–14].

Based on these findings, we investigated the possible association between vasomotor function of the epicardial and microvascular coronary vasculature, expression/activation of pro-inflammatory cytokines and the NO pathway in 42 cardiac transplant recipients in an early but stable phase after HTx.

Materials and methods

The study protocol was reviewed and approved by the Human Subjects Research Committee of the Ludwig-Maximilians University and all participants granted written informed consent. Cardiac transplant recipients ($n = 41$) were chosen for transplantation by donor dependent clinical criteria such as weight, height and blood group, exclusively.

Post-operative exclusion criteria were impaired renal function (serum creatinine levels > 1.6 mg/dl), acute signs of infection 10 days or less prior to cardiac catheterization and blood sample collection, rejection grade ≥ 1 b International Society for Heart and Lung Transplantation (ISHLT) 10 days or less prior to collection of biopsy samples and the presence of coronary artery disease (CAD).

Patients were kept on standard immunosuppressive therapy, which included either tacrolimus (FK 506) in combination with mycophenolate mofetil or cyclosporine with azathioprine and prednisolone (Table 1). Other medication consisted of calcium antagonists, ACE (angiotensin converting enzyme)-inhibitors, diuretics and lipid-lowering drugs. None of the patients received long-acting drugs and, except for the immunosuppressive regimens, all medications were discontinued 12–24 h prior to data and sample collection.

Blood samples for measurement of total nitrite and cytokines were withdrawn from the coronary sinus before endomyocardial biopsies were taken and from the aortic root prior to assessment of the coronary vasomotor function in the epicardial and microvascular compartments.

Endothelium dependent and independent epicardial vasomotion

Quantitative coronary angiography with a computerized automatic analysis system (Hicor, Siemens) was used to assess the vasomotor response (epicardial luminal diameter changes, %) of the coronary arteries in an early but stable phase after HTx (37 ± 5 days post HTx). Proximal and distal coronary artery segments identified between easily visualized branch points were selected for analysis in the left anterior descending and circumflex coronary arteries. The mean proximal and distal diameter was calculated for each coronary segment from a series of at least two measurements.

Endothelium dependent vasomotor response was measured with intracoronary administration of acetylcholine (1.0 and 30.0 µg/min for 5 min each).

Endothelium independent vasomotion was determined with intracoronary infusion of adenosine (80.0 and 160.0 µg/min for 5 min each) and intracoronary bolus application of 0.2 mg nifedipine. Data were expressed as percentage of the difference between stimulated and baseline luminal diameter of the proximal and distal segments.

Endothelium dependent and independent microvascular vasomotion

Microvascular vasomotor response was assessed by flow velocity measurements with an intracoronary Doppler flow wire [0.018 in. (0.04 cm); Flo Wire, Cardiometrics]. The flow wire was introduced in a 6-F Judkins catheter and positioned in the proximal part of the left anterior descending or circumflex coronary artery. The technical details of the system and its validation for accurate measurements have been described in detail elsewhere [15]. After successful positioning of the flow wire, yielding a stable and optimal velocity tracing, baseline flow velocity readings were obtained. Hyperemic flow velocity data were determined with intracoronary adenosine infusion (Ad; 80.0 and 160.0 µg/min over 5 min each). Endothelium dependent changes in flow velocity were measured with intracoronary acetylcholine (iAch; 1.0 and 30.0 µg/min over 5 min each). Finally, a long-acting bolus of nifedipine was given i.c. to obtain endothelium independent vasomotor information in addition to adenosine. Coronary flow velocity reserve was assessed by calculation of the ratio of peak (Ad, Ach or nifedipine) to base-

Table 1 Donor and recipient demographics. Patient demographics are shown with respect to pre- and post-transplant clinical criteria of recipients as well as donor-dependent criteria relevant to post-transplant allograft function. No significant differences are noted with regard to the parameters evaluated (*CFVR* coronary flow velocity reserve, *CMV* cytomegalovirus, *CAD* coronary artery disease, *PVR* pulmonary vascular resistance, *Dx* Diagnosis, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins)

	Impaired CFVR ($n = 11$)	Normal CFVR ($n = 31$)
Donors		
Age (years)	36.2 ± 15.5	31.6 ± 11
Weight (kg)	73.7 ± 10.5	74.6 ± 12.1
Height (cm)	174.3 ± 7.7	173 ± 9.9
Sex (% male)	54.4	51.6
CMV positive (%)	45.4	50.1
Graft ischemic time (min)	223 ± 39	197 ± 49
Total bypass time (min)	95.7 ± 22	117 ± 29.8
Weaning time from bypass (min)	33.5 ± 8.5	36.9 ± 14.3
Recipients		
Age (years)	56.8 ± 8.8	47.7 ± 10.6
Weight (kg)	70.8 ± 13.9	75.6 ± 10
Height (cm)	172 ± 7	177 ± 8.7
Sex (% male)	82	93
CMV positive (%)	36	29
CMV mismatch, *n* [%]	5 [45.5]	15 [48.3]
Pre-transplant Dx (% CAD)	36.6	35.4
PVR (pre-transplant; Wood units)	1.89 ± 0.7	2.46 ± 1.1
Study examination (days after HTx)	34.5 ± 10.6	38.1 ± 12.4
Tacrolimus (ng/ml)	15.6 ± 4.1	16.1 ± 3.3
Creatinine (mg/dl)	1.36 ± 0.2	1.2 ± 0.3
Cholesterol (mg/dl)	180.1 ± 16.3	200.3 ± 23.6
LDL (mg/dl)	104.5 ± 22.6	125 ± 29.3
HDL (mg/dl)	47.9 ± 6.3	48.7 ± 18
Lp(a) (mg/dl)	21.6 ± 15.8	32.8 ± 13.1
Native CAD risk factors		
Diabetes, *n* [%]	2 [18.1]	4 [12.9]
Hypertension, *n* [%]	2 [18.1]	6 [19.3]
Hyperlipidemia, *n* [%]	5 [45]	16 [51.6]
Tobacco, *n* [%]	2 [18.1]	7 [22.5]
Hyperuremia, *n* [%]	1 [9]	4 [12.9]
Alcohol, *n* [%]	1 [9]	2 [6.4]
Number of CAD risk factors per patient	1.27	1.35
Immunosuppressive regime, *n* [%]		
Tacrolimus + mycophenolate + prednisolone	8 [72]	21 [67.7]
Tacrolimus + azathioprine + prednisolone	1 [9]	4 [12.9]
Cyclosporine + azathioprine + prednisolone	2 [18.1]	6 [19.3]

line blood flow velocity. Heart rate, mean arterial pressure, coronary flow velocity and electrocardiogram were monitored continuously throughout the procedure. It was assured that epicardial vasoconstriction in response to iAch did not alter the flow velocity reserve of the microvascular bed.

Determination of total plasma nitrite and cytokines

Plasma of aortic root and coronary sinus blood samples was stored frozen at –80 °C. Total nitrate/nitrite levels were measured by the Gries reaction. In brief, plasma was deproteinized by ultrafiltration (Centrifree micropartition system, Amicon). The nitrate content of the sample was reduced to nitrite with a nitrate reductase. Sample aliquots were incubated in the presence of 0.1 U/ml nitrate reductase in 2.5 mM phosphate buffer containing 5.0 µM flavin adenine dinucleotide (FAD) and 50 µM reduced nicolineamil dinuclertid phosphat (NADPH) (all from Boehringer-Mannheim) in final sample volume of 106.7 µl and incubated for 30 min at 37 °C. NADPH was oxidized by adding 6.7 µl of a reaction mix containing lactatdehydrogenase [dilution 1:10 (60 µl)] and pyruvate (140 µl) followed by 5 min incubation at 37 °C. Finally the Gries reagent was added followed by incubation at 37 °C for 10 min. The reaction was stopped by adding 333.0 µl of cold trichloric acid (TCA) (1.2 M). Samples were measured by spectrophotometric analysis at 540 nm. A standard curve, was performed in each experiment. The nitrite content of the samples was calculated from the standard curve, which was linear within this range.

The pro-inflammatory cytokine TNF-a and its specific receptors TNF-Rp1, TNF-Rp2 as well as IL-6 were measured by commercially available enzyme immunoassay (EIA) and enzyme linked immunoabsorbant assay ELISA as described [4].

RNA extraction and cDNA preparation

Myocardial biopsy samples were taken for determination of cardiac gene expression. Samples were immediately frozen in liquid nitrogen and stored at –80 °C. For RNA extraction, samples were homogenized with OMNI 200 homogenizer (Süd-Laborbedarf, Ga-

Table 2 Primer sequence. The primers used in this study are shown with their specific sequences. It was assured that the design of the various primers resulted in specific detection of the product and not of genomic DNA (*GAPDH* glycerolaldehyde-3-phosphate dehydrogenase, *ecNOS* endothelial constitutive nitric oxide synthase, *iNOS* inducible nitric oxide synthase, *bp* base pairs)

Primer	Sequence 5′ to 3′	Product size (bp)
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT and CATGTGGGCCATGAGGTCCACCAC	983
iNOS	GGCCTGGAAACGCACAAGCTG and TTGGGGTTGAAGGCACAGCTG	506
ecNOS	GAAGAGGAAGGAGTCCAGTAACAC and GGTGGCCCTCGTGGACTTGCTG	451

uting) in 600 µl of lysis buffer (Quiagen, Hilden). Insoluble material was separated from the lysate by centrifugation at 10,000 g for 3 min. Total RNA was extracted from the supernatant using spin columns with a selective binding silica-based membrane (RNeasy Kit, Quiagen, Hilden). The total RNA was quantified by measuring the optical density at A_{260} and confirmed by gel electrophoresis. Complementary DNA (cDNA) was prepared from 2 µg of total RNA in 30 µl reverse transcription buffer (Gibco BRL, Paisley) supplemented with 0.6 mM each of dATP, dGTP, dCTP and dTTP (all New England Biolabs, Schwalbach), 32 U RNase inhibitor (Boehringer, Mannheim), 400 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Gibco BRL, Paisley), 10 mM dithiotreitol, and 1.5 µM $p(dt)_{15}$ primer (Boehringer, Mannheim) at 37 °C for 60 min. Subsequently, the reaction mixture was heat-inactivated for 10 min at 95 °C.

Polymerase chain reaction (PCR) procedure

An aliquot (3 µl) of cDNA was amplified by PCR with a DNA thermal cycler (Perkin Elmer 480, Cetus Corp, Norwalk, Conn.). The amplification reaction was carried out in a total volume of 50 µl of PCR buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 50 mM KCl, 200 µM each of dATP, dGTP, dCTP and dTTP, 400 nM each of 3' and 5' primers, and 1 U Taq DNA polymerase (Boehringer, Mannheim). The oligonucleotide primers had been synthesized on a DNA synthesizer (Applied Biosystems, Norwalk, Conn.) and purified using Sephadex columns (NAP 5, Pharmacia, Freiburg). The primers were designed such that the expected products were only obtained from cDNA, but not from genomic DNA. The GAPDH (glycerolaldehyde-3-phosphate dehydrogenase) transcript was used as an internal control of the processed RNA. The nucleotide sequences of the primers used in the amplification reaction are shown in Table 2. The PCR reaction mixture was covered with 50 µl of light mineral oil (Sigma Chemie, Deisenhofen). After 1 min pre-denaturation at 94 °C, the PCR conditions were as follows: denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 1 min. To ensure detection of low-abundance mRNA, 35 amplification cycles were performed. A sample of 10 µl of each amplified product was subjected to electrophoresis in a 1% agarose gel (Promega, Madison, Wisc.), stained with ethidium bromide and visualized by UV illumination.

Semiquantitative analysis was performed using a densitometric analysis system. Each signal was normalized for the DNA standard of each gel to account for variances between gels. In addition, normalization for the housekeeping gene GAPDH was performed to account for the variability of sample quality.

Statistical analysis

Group data are presented as arithmetic mean values ± SD. One way analysis of variance (ANOVA) was performed for comparison of functional parameters. Paired *t*-test was used to assess differences between each individual. Correlation coefficient was determined by simple regression analysis. *P*-values of 0.05 or less were considered statistically significant.

Results

All patients presented with normal renal function parameters (serum creatinine 1.1 ± 0.3 mg/dl) and absence of acute rejection episodes of grade ≥ 1b (ISHLT) at least 10 days prior to examination. In addition, no signs of acute bacterial or viral infection were observed or had to be treated at the time of investigation or within 10 days prior to examination.

As shown in Table 1, no significant differences were noted between patients with normal and those with impaired CFVR with respect to donor or recipient demographics. Moreover, ischemic time, preservation solution and conventional risk factors for atherosclerosis did not differ significantly between the two groups. This is of importance since, if present, these pro-inflammatory factors might have accounted for differences in endothelial function, cytokine and nitric oxide production.

Endothelium dependent and independent microvascular and epicardial vasomotor function

In 11 patients (26.1%, group A) a significantly impaired flow increase to the endothelium dependent vasodilator acetylcholine was observed, suggesting a microvascular endothelial dysfunction (CFVR: 1.6 ± 0.5 increase, $P = 0.003$). Thirty-one patients showed normal endothelium dependent coronary flow velocity reserve (CFVR: 3.0 ± 0.7 increase; group B) (Fig. 1). Between the two groups of patients, no significant differences were found with respect to epicardial endothelium dependent (prox. segments: group A: –4.8 ± 1.8% vs group B: –6.1 ± 1.5%, P = ns; distal segments: group A: –11.7 ± 2.1% vs group B: –8.3 ± 2.4%, P = ns) or independent vasomotor function (prox. segments: group A: 6.2 ± 1.7% vs group B: 7.6 ± 1.8%, P = ns; distal segments: group A: 18.7 ± 6.8% vs group B: 16.4 ± 5.3%, P = ns). None of the patients presented with angiographic evidence of macroscopic CAD. In addition, averaged mean and maximal intimal thickness as assessed by intracoronary ultrasound did not differ significantly between the two groups (data not shown).

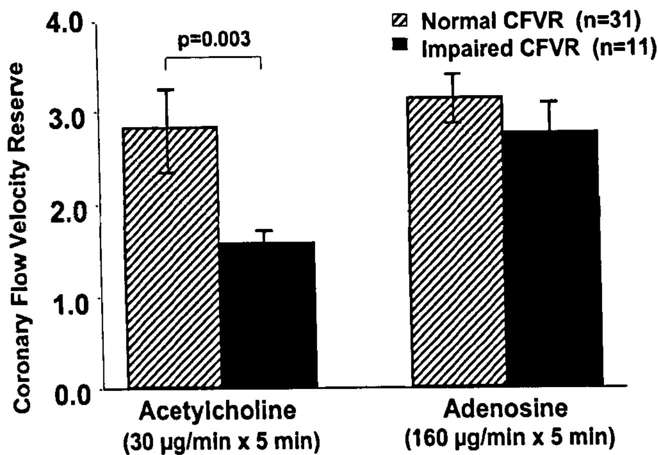

Fig. 1 Microvascular coronary flow velocity reserve (CFVR) in response to endothelium dependent (acetylcholine; Ach) and endothelium independent (adenosine) stimuli. In 26.1% of patients an impaired response to Ach was observed (1.65 ± 0.23 increase vs 3.0 ± 0.7 increase, $P = 0.003$). However, no significant differences with respect to adenosine were observed between the two groups

Myocardial gene expression for cNOS and iNOS

No significant differences were noted between the two groups with respect to myocardial iNOS gene expression. In addition, a comparable score for cNOS gene expression was observed (Fig. 2A).

Transcardiac levels of plasma nitrite and cytokines

In up to 60% of all patients a significant transcardiac release of cytokines was observed (IL-6: 58.8%; TNF-Rp2: 58.8%; TNF-a: 53.3%; TNF-Rp1: 41.6%). In addition, patients with an impaired CFVR to Ach showed higher levels for IL-6 and TNF-a in the coronary sinus when compared to those with normal CFVR (Fig. 2B). Coronary sinus plasma nitrite levels were higher in patients with impaired CFVR (Fig. 2B). In addition, a significant transcardiac nitrite production was observed in patients with impaired endothelium dependent CFVR, suggesting transcardiac nitric oxide production (Fig. 2C). In this group, CS nitrite levels correlated significantly with CS TNF-α concentration (Fig. 2D), indicating cytokine induced cardiac nitric oxide production.

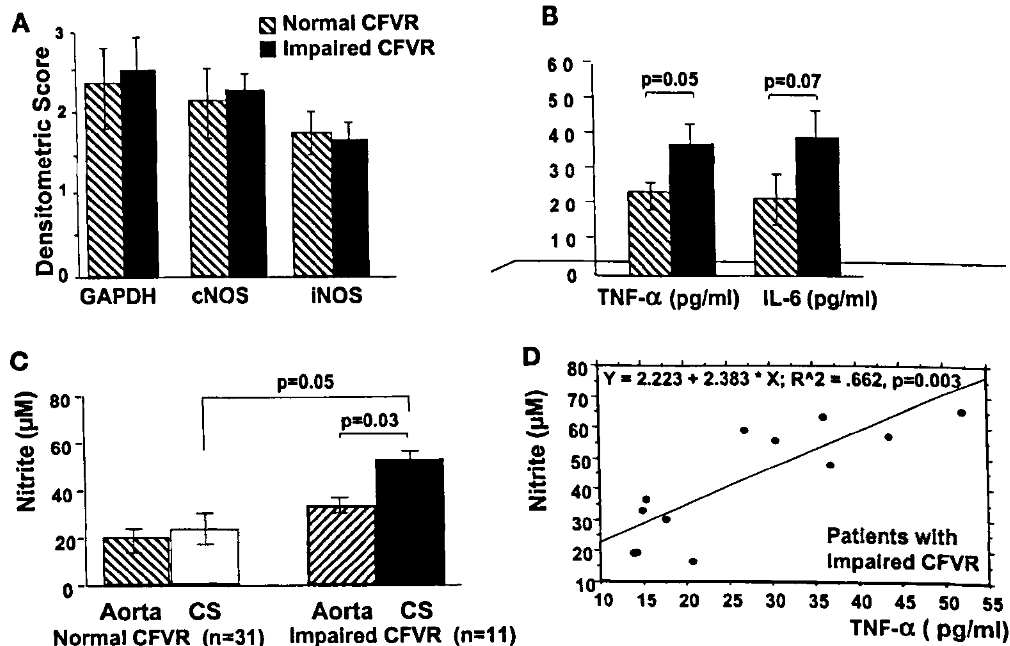

Fig. 2 **A** Semiquantitative determination of myocardial gene expression for constitutive and inducible nitric oxide synthase (cNOS, iNOS) did not reveal significant differences between the two groups. However, in all patients examined, iNOS expression was observed. **B** A significantly higher TNF-α level (pg/ml) was found in the coronary sinus (CS) in patients with an impaired CFVR to Ach 37 ± 5 days after cardiac transplantation (22.1 ± 4.8 pg/ml vs 37.2 ± 5.8 pg/ml, $P = 0.05$). In addition, IL-6 (pg/ml) levels in the CS tended to be higher in these patients (21.4 ± 8.1 pg/ml vs 39.1 ± 8.4 pg/ml, $P = 0.07$). **C** A significant transcardiac nitric oxide production was observed in patients with impaired CFVR to Ach measured as the difference between aortic and CS levels of the stable end-product nitrite; group A (impaired CFVR): aorta 43.9 ± 3.7 µM, vs CS 52.8 ± 5.6 µM, $P = 0.05$; group B (normal CFVR): aorta 24.1 ± 5.2 µM vs CS 32.3 ± 6.4 µM, P = ns). **D** In patients with an impaired CFVR to Ach a significant correlation between CS, TNF-a (pg/ml) and nitrite levels (µM) were observed, indicating a cytokine-induced cardiac nitric oxide production in this group of patients

Discussion

The results of the present study indicate that 26.1% of patients have an impaired endothelium dependent microvascular CFVR in response to Ach 37 ± 5 days after HTx. This finding may be of importance, since recent data suggest that endothelial dysfunction in epicardial coronary arteries early after HTx predicts the development of TxCAD visible at 1 year post-transplant [6].

A possible relationship between endothelial dysfunction and expression and activation of vasoactive mediators early after HTx has not been addressed to date. The new finding of the present study is the transcardiac nitrite production in patients with impaired CFVR, indicating cardiac nitric oxide production. In addition, iNOS and cNOS gene expression was observed in both patient groups and did not differ between groups. Which isoenzyme actually produced NO within the heart remains therefore uncertain. However, increased iNOS activation occurs in the absence of enhanced gene expression and may be a potential source for cardiac NO production. Supportive for this notion is the association between coronary sinus nitrite and TNF-α levels in patients with impaired CVFR. It is known that the inducible NO pathway is activated by pro-inflammatory cytokines such as TNF-α or IL-6 [16]. iNOS-derived NO may deprive the endothelium of substrate for NO production and might explain the compromised endothelium dependent vasodilatation observed in approximately one-third of the patients. Similar findings have been shown recently in a rabbit model of atherosclerosis [17–19].

In addition, up-regulation of iNOS activity has been shown in acute cardiac allograft rejection. Selective modulation of this isozyme by aminoguanidine prolonged graft survival and prevented vascular barrier dysfunction in rats [11, 17]. Moreover, Lewis et al. showed an association between vascular and contractile dysfunction of the left ventricle and induction of iNOS in the human cardiac allograft [13].

On the other hand, expression and activation of iNOS may also contribute to beneficial long-term effects with regard to TxCAD. Koglin et al. have demonstrated that NO derived from iNOS is deleterious in the setting of acute rejection episodes early after HTx; they also showed that iNOS exerts protective long-term effects by reducing smooth muscle cell proliferation and migration, thereby preventing neointima formation and the progression of TxCAD in iNOS knockout mice [20]. Similar observations were reported by others in a model of adenovirus-mediated iNOS gene transfer into rat allografts [21].

In the present study, endothelial cNOS gene expression appeared to be comparable in the two groups. Similar findings were reported in an experimental setting by Akyürek et al. [22]. However, the functional assessment revealed an impaired response of the microvasculature to Ach. Decreased endothelium derived NO (or its increased degradation) may contribute to endothelial dysfunction due to enhanced leukocyte and platelet adhesion to the vascular wall [23–25]. This is supported by Drexler et al., who reported beneficial effects of intracoronary L-arginine in cardiac transplant recipients with impaired blood flow response to Ach, supporting the notion that endothelial dysfunction in those patients was improved by administration of the nitric oxide precursor [26]. Moreover, decreased cNOS activity may enhance the expression of adhesion molecules, contributing to further endothelial cell injury and subsequent development of TxCAD. In fact, it has been shown recently that enhanced arteriolar endothelial ICAM-1 expression correlated with early angiographically visible TxCAD in humans [1, 27].

In conclusion, the present data indicate that approximately 26% of patients develop microvascular endothelial dysfunction within 1 month after cardiac transplantation. The association of vasoactive and immunomodulatory mediators including nitric oxide, TNF-a and IL-6 in this group of patients may be at least in part responsible. Even though causation needs further investigation in longitudinally designed studies, the contribution of these vasoactive factors to the development of endothelial dysfunction in the early phase after HTx may be of therapeutic interest in these patients.

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