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ORIGINAL ARTICLE

Cold ischemia contributes to the development of chronic rejection and mitochondrial injury after cardiac transplantation

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Summary

Chronic rejection (CR) remains an unsolved hurdle for long-term heart transplant survival. The effect of cold ischemia (CI) on progression of CR and the mechanisms resulting in functional deficit were investigated by studying gene expression, mitochondrial function, and enzymatic activity. Allogeneic (Lew → F344) and syngeneic (Lew → Lew) heart transplantations were performed with or without 10 h of CI. After evaluation of myocardial contraction, hearts were excised at 2, 10, 40, and 60 days for investigation of vasculopathy, gene expression, enzymatic activities, and mitochondrial respiration. Gene expression studies identified a gene cluster coding for subunits of the mitochondrial electron transport chain regulated in response to CI and CR. Myocardial performance, mitochondrial function, and mitochondrial marker enzyme activities declined in all allografts with time after transplantation. These declines were more rapid and severe in CI allografts (CR-CI) and correlated well with progression of vasculopathy and fibrosis. Mitochondria related gene expression and mitochondrial function are substantially compromised with the progression of CR and show that CI impacts on progression, gene profile, and mitochondrial function of CR. Monitoring mitochondrial function and enzyme activity might allow for earlier detection of CR and cardiac allograft dysfunction.

Introduction

Annual heart allograft failure rates about 3–5% and the main reason for graft loss after the first year is chronic allograft vasculopathy – also termed chronic rejection (CR) [1]. Although life saving when needed, the organ half-life after retransplantation is inferior when compared with primary heart transplantation [2]. Myointimal hyperplasia results in an ischemic heart disease, and the lack of graft innervation and angina-pectoris symptoms allows ventricular arrhythmias, sudden cardiac death, or heart failure to occur without warning. Diagnostic tools, such as endomyocardial biopsy, coronary angiography or intracoronary ultrasound, help to identify the extent of

vascular luminal narrowing but fail to predict the individual risk for myocardial dysfunction [3–6].

In addition to alloimmune-mediated mechanisms, non-immunological factors such as cold ischemia (CI), donor age, metabolic factors and hypertension have been shown to contribute to the development of CR [7–10]. CI induces an inflammatory response leading to pathological remodeling of graft vessels and might be particularly relevant for induction and progression of CR [11,12].

Mitochondria are recognized as a critical organelle for long-term survival of cardiac allografts. Cold and warm ischemia, but also acute rejection, has been shown to impact mitochondrial function [13–19]. We have previously found that CI results in specific defects of respiratory complexes (e.g. complex I), reduced respiratory function, rupture of the outer mitochondrial membrane, and cytochrome *c* release in cardiac transplantation correlating with the loss of cardiac contractile function [20,21].

In this study, we have used gene expression analysis as a screening tool for elucidating the molecular events promoting CR in a rat heart transplant model and investigated the impact of ischemia/reperfusion overlaying the alloimmune response. Mitochondrial function and different segments of mitochondrial electron transport chain were then investigated in permeabilized myocardial fibers. In summary, our findings indicate that prolonged cold ischemia has a deleterious effect on the progression of CR, cardiac function, and mitochondrial damage. Alterations in gene expression and mitochondrial homeostasis and loss of respiratory and enzymatic capacities may contribute to myocardial dysfunction in CR.

Materials and methods

Animal care

Inbred male adult F344 (RT1) and Lewis ($RT1^l$) rats (200–250 g) were obtained from Harlan CPB (Austerlitz, Germany). Animals received standard laboratory diet with unlimited access to water. Environmental conditions and human care were in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society of Sciences and published by the National Institute of Health (NIH Publication Vol. 25, No. 28 revised 1996) as well as national laws. Anesthesia was induced through intraperitoneal injection of 0.5 ml/kg sodium pentobarbital (Veterinaria AG, Austria).

Animal model and experimental design

Cardiac transplantations were carried out in the Lewis to F344 rat model employing a microsurgical procedure [22]. Before excision, hearts were flushed retrogradely with 10 ml of cold (0–1 °C) University of Wisconsin solution (UW; ViaSpan®, Du Pont Pharmaceuticals, Wilmington, DE, USA) via the ascending aorta. After retrieval, hearts were either transplanted immediately or exposed to 10 h of ischemia by storage in 100 ml of cold (0–1 °C) preservation solution and then transplanted. Recipients of allografts were injected with Cyclosporin A (CsA, 5 mg/kg/day i.m.; Novartis, Basel, Switzerland) for 10 days to prevent early acute rejection. Findings from a previous trial had revealed that progressive acute rejection with graft loss can be prevented with this therapy, whereas chronic rejection developed within 60 days [23]. Allografts transplanted immediately (CR) or after 10 h of cold ischemia (CR-CI; $n = 5$ per group) were compared with corresponding syngeneic control groups (SYN no ischemia, SYN-CI 10 h cold ischemia, $n = 3$ per group) (Fig. 1).

Cardiac performance was monitored daily by palpation and graded per a previously described scoring scheme (1: No contraction at palpation and minimal function at inspection upon laparotomy, 2: weak or partial contractions detectable by palpation, 3: homogenous contraction of the ventricle at low intensity (frequency or amplitude), 4: normal atrial and ventricular contraction intensity on days 2, 10, 40, and 60 days after transplantation [20,21]. Hearts were then excised and sagittal sections placed in formalin or snap frozen in liquid nitrogen. For sample storage in preparation of mitochondrial function studies, a specific cryopreservation protocol was applied [24]. Thin bundles of myocardial fibers were placed in DMSO-based cryopreservation medium and immediately frozen in liquid nitrogen.

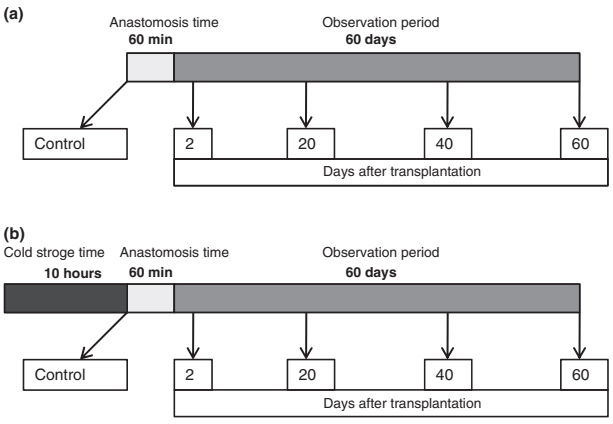

Figure 1 Experimental protocol. (a) Grafts transplanted with no cold ischemia time in syngeneic and allogeneic rat strain combinations (SYN and CR). (b) Syngeneic and allogeneic grafts transplanted after 10 h of ischemia. After assessment of function, grafts in all groups were retrieved at 2, 10, 40, and 60 days after transplantation.

Histology

Paraffin-embedded sections showing both ventricles were stained with hematoxylin/eosin (HE) and evaluated with respect to five criteria for chronic rejection: vasculitis, interstitial cellular infiltration, edema, myointimal proliferation, and fibrosis. The percentage of affected vessels and the degree of luminal occlusion by myointimal proliferation were graded for each vessel (grade 0: no occlusion, grade 1: occlusion <25%, grade 2: occlusion 25–50%, grade 3: occlusion >50%) and a mean vessel score per sample and group was then calculated. Images were taken using a CCD camera (Kappa Instruments, Munich, Germany) mounted on an Olympus microscope.

Tissue preparation, RNA isolation, microarray hybridization, and real time PCR

Total RNA was isolated from each heart using TriReagent according to manufacturers' instructions (Molecular Research Center; MRC, Cincinnati, OH, USA) and further purified by treating it with DNAse and protease K using High Pure RNA Tissue Kit (Ambion, Huntington, UK). Integrity of RNA was quantified in a photometer and analyzed by gel electrophoresis.

For microarray analysis, equal amounts of highly purified RNA from five (allogeneic) or three (syngeneic) hearts per group were pooled to minimize variations among animals. Sample preparation, labeling, and hybridization were performed according to the MIAME guidelines and are summarized in supplemental Table 1 legend [25]. Regulated genes were selected by a greater than fourfold difference in fluorescence signal. Data were then transferred to Genesis [26] and analyzed by hierarchical clustering [27].

Gene expression of selected genes in samples from individual animals (not pooled) was performed employing quantitative real time PCR on an IQ5 Instrument (BioRad, Hercules, CA, USA) and according to MIQE guidelines [28]. In all, 1 μg total RNA was reversely transcribed using the SuperScript First-Strand Synthesis System from Invitrogen (Carlsbad, CA, USA) (Cat. No.: 11904-018) according to the supplier's instructions. Primer and Probe sets for rat tubulin alpha, ATP synthetase subunit 6 (ATP 6), NADH dehydrogenase subunit 4 (NADH 4), Cytochrome *c* oxidase 3 (COX 3), cytochrome *c* oxidase subunit VIII-h (heart muscle) (COX 8h), and global ischemia inducible protein (GIIP) were obtained from Exiqon (Copenhagen, Denmark). PCR assays contained a volume of 25 μl, 10 ng cDNA template, 20 μM primer, and 10 μM probe. PCR was carried out with a 10 min hot start activation (94 °C) followed by 40 cycles of 20 s denaturation (94 °C) and 1 min annealing/extension at 60 °C. A dissociation analysis (60–95 °C) was performed to confirm identity and purity. LinReg PCR calculated the efficiency of each reaction by measuring the rate of the reaction in the log-linear portion of the fluorescence versus cycle function. Reaction efficiency was expressed as a value between 1 and 2 [2: perfect doubling of the amplification with each cycle (100% efficiency), 1: no amplification (0% efficiency)]. PCR reaction was optimized to obtain reaction efficiencies between 1.9 and 2. Quantification was carried out using the ΔΔCt method and read relative to mRNA pooled from seven naive rat hearts [29]. The Ct values were normalized to alpha tubulin as housekeeping gene.

High resolution respirometry of permeabilized myocardial fibers

Permeabilized myocardial fibers isolated from the grafts were used for functional assessment of mitochondria *in situ* following a previously published technique [30]. Samples from five animals in each allogeneic and three in each syngeneic group were analyzed. Respiration was measured twice for each sample and a mean was calculated. Myocardial fibers were isolated by dissection of the left ventricle in relaxing solution on ice and permeabilized with saponin. Fibers were then frozen in liquid nitrogen, using DMSO-based cryopreservation medium [24]. Mitochondrial function was measured by high resolution respirometry at 30 °C, using two-channel titration-injection respirometer (Oroboros Oxygraph, Innsbruck, Austria) with a respiration medium consisting of 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid free, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1. DatLab software (Oroboros, Innsbruck, Austria) was used for data acquisition and analysis. A substrate/inhibitor titration protocol was applied to test for multiple mitochondrial defects [15,30]. Respiration was stimulated by 1 mM ADP (state 3) and measured sequentially through complex I (10 mM glutamate, 5 mM malate), complex II (10 mM succinate, 0.5 μM rotenone), and complex IV (0.5 mM TMPD, 2 mM ascorbate, 5 μM antimycin A). Chemical background controls were used to correct for the auto-oxidation of TMPD and ascorbate. Respiration with TMPD + ascorbate was measured also after stimulation by added cytochrome *c* (10 μM, Boehringer Mannheim, Germany).

Enzyme activities

Frozen tissue samples (40–100 mg) were minced, placed into ice-cold 0.1 M phosphate buffer, pH 7.4 at 25 °C, homogenized for 30 s, and used for enzyme assays. The activity of mitochondrial enzymes NADH:ubiquinone oxidoreductase (complex I), cytochrome *c* oxidase (complex

IV, COX), citrate synthase, and cytosolic enzyme lactate dehydrogenase (LDH) was measured spectrophotometrically at 30 °C as described previously [30]. Enzyme activities were measured twice for each sample.

Statistical data analyses

All data are presented as mean ± SD. Histology, respirometric, and enzymatic data were analyzed by ANOVA followed by the Tukey's test for pair-wise comparison between individual groups. Significance was considered at $P < 0.05$.

Results

Graft survival and cardiac function (score)

Administration of Cyclosporin A (5 mg/kg) prevented acute rejection and early graft loss. Cardiac score at 10 days after transplantation (3.2 ± 0.45) was significantly lower in allogeneic groups undergoing CI when compared with all other groups at this time point ($P < 0.01$, Fig. 2). At 40 days, organ function in both allogeneic groups (CR and CR-CI) was significantly lower than in syngeneic controls ($P < 0.01$, Fig. 2). The decrease was significantly more pronounced in allografts undergoing CI (2.1 ± 0.9 for CI-CR vs. 3.0 ± 0.0 for CR, $P < 0.05$). Cardiac performance further declined at 60 days after transplantation to scores of 1.6 ± 0.49 (CI-CR group) and 1.86 ± 0.84 (CR group). Cold ischemia also resulted in a reduced organ function at 40 and 60 days in isografts, but the decline did not reach statistical significance (Fig. 2). Isografts transplanted without ischemia remained unaffected during the entire observation period.

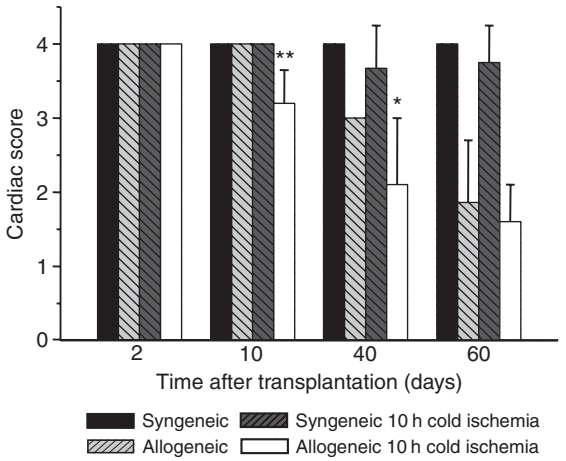

Figure 2 Contractile cardiac performance (cardiac score). Graft function decreased significantly at day 40 after allotransplantation, whereas it remained unchanged in syngeneic grafts. Decline in myocardial performance as assessed with a previously established grading scale was more pronounced in allografts undergoing 10 h of cold ischemia (CI) prior to transplantation.

Histology

Transplant vasculopathy together with interstitial and perivascular inflammatory infiltrates and myocardial fibrosis were evident in allogeneic groups at 40 and 60 days after transplantation (Fig. 3). Intimal thickening comprising smooth muscle cell proliferation progressed gradually with time in both allogeneic groups CR and CR-CI. However, in hearts exposed to cold ischemia, changes were significantly more pronounced at 40 days after transplantation (1.22 ± 0.81, $n = 64$ vs. 0.70 ± 0.87, $n = 93$, $P = 0.0002$) and 60 days (2.11 ± 0.81, $n = 72$ vs. 1.62 ± 1.05, $n = 104$, $P = 0.001$; also see Fig. 3). In syngeneic grafts (SYN and SYN-CI), cold ischemia had only a marginal effect on the degree of myointimal proliferation at 60 days (0.31 ± 0.74, $n = 26$ vs. 0.06 ± 0.25, $n = 16$, $P = 0.633$, NS). However, the percentage of affected vessels in syngeneic groups ranged from 6.3 ± 0.0% (at days 60, without CI) to 20 ± 0.0% and 19.2 ± 5.4% at days 40 and 60 in the groups exposed to 10 h cold ischemia. In contrast, 97.2 ± 6.9% and 82.7 ± 5.7% of vessels were affected at 60 days in allografts transplanted with and without ischemia, respectively.

Gene expression studies

Hierarchical cluster analysis revealed a cluster containing several genes of mitochondrial respiratory chain, such as subunits of complexes I, IV, and V regulated when hearts were exposed to CI (see supplemental Tables S1 and S2 for details of gene expression early and late after transplantation). The pattern of expression for this subset differed both early after transplantation as well as late (40 and 60 days) after transplant. The regulated 'genes of interest' was both mitochondrially and nuclearly encoded, hence mitochondrial DNA damage was not considered the sole reason for gene regulation.

A representative number of most affected genes were selected for further evaluation by qPCR. The gene set included three typical mitochondrially encoded subunits of mitochondrial oxydative phosphorylation system: NADH 4, COX 3, ATPase 6, nuclear encoded mitochondrial COX-8H, and the nuclear encoded global ischemia-induced protein GIIP also known as endoplasmic reticulum oxidoreductin-1 (Ero1-L), respectively. When analyzed by qPCR, variations among the different samples within a group were very low, and the overall fold increase/decrease of gene transcription was in good agreement with results obtained by microarray-based gene expression studies. CI resulted in a profound change (upregulation) in the expression of this

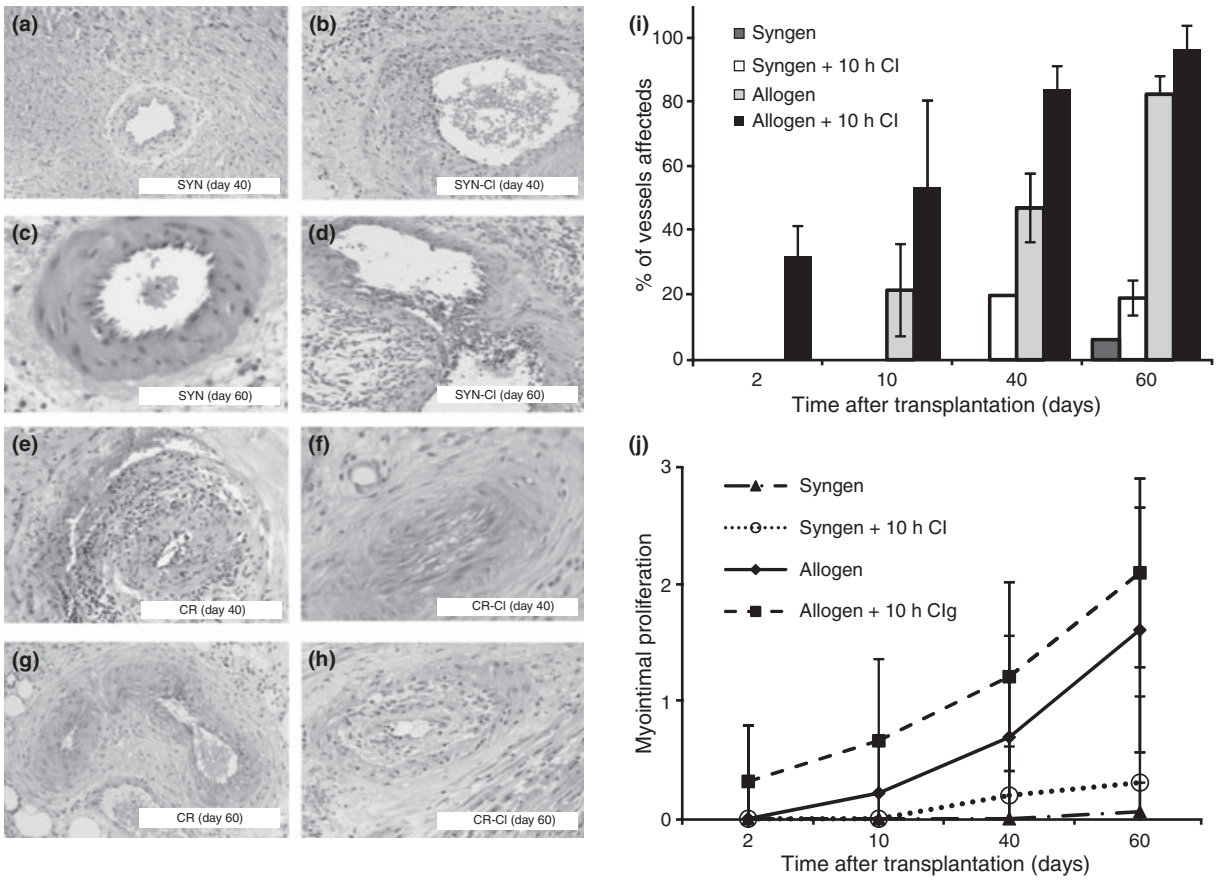

Figure 3 Histological assessment of CR in cardiac iso- and allografts. Transplant vasculopathy, interstitial and perivascular inflammatory infiltrates, and myocardial fibrosis were assessed and graded in all groups. (a–d) Syngeneic controls. At 40 days, myointimal proliferation was significantly more pronounced in CR hearts exposed to 10 h of cold ischemia (CI) prior to transplantation (f versus e). At 60 days, the difference was less stringent (g and h), but still significant. In syngeneic grafts, CI had a marginal effect on myointimal proliferation at 60 days after transplantation (0.31 ± 0.74 vs. 0.06 ± 0.25 for SYN-CI and SYN, respectively, $P = 0.128$, NS) with the percentage of affected vessels ranging from 6.3 ± 0.0% (no CI) to 20 ± 0.0%. (i) In allografts, 97.2 ± 6.9% (CI) and 82.7 ± 5.7% (no CI) of vessels were affected at 60 days. (j) Myointimal proliferation increased with reperfusion time. This increase was more pronounced in CR-CI.

set of genes in isografts as well as allografts (Fig. 4). Interestingly, prolonged cold ischemia prior to transplantation resulted in an increased expression of ATP 6, COX 3, COX 8h, and NADH 4 in both iso- and allografts at almost all time points. In organs transplanted without cold ischemia, this set of genes was found down regulated (except for COX 8h at 2 and 10 days after transplantation) (Fig. 4), indicating a specific and significant effect of prolonged cold ischemia on mitochondrial gene expression. GIIP/Ero1-L was expressed at high level at all time points in heart transplants after transplantation independent of cold ischemia.

Mitochondrial respiratory function and activity of marker enzymes

Mitochondrial functional parameters were tested with different respiratory substrates, electron donors, and specific inhibitors for step-by-step assessment of different segments of the mitochondrial respiratory chain to detect and localize defects in respiratory complexes involved in mitochondrial injury. Mitochondrial respiratory function (maximal ADP-stimulated respiration at saturated substrates and ADP concentrations) was preserved in isografts transplanted without cold ischemia and was significantly less affected in cold stored isografts compared with corresponding allografts. Mitochondrial active (state 3) respiration drastically declined in a time-dependent manner (time after transplantation) in CR and CR-CI groups with all substrates used for the analysis, indicating a general loss of mitochondrial capacity (Fig. 5a–d). This decline was faster and significantly more severe in CR-CI than in CR. At day 2, mitochondrial respiration was similar in CR-CI and CR and was not significantly different from corresponding syngeneic groups. At day 10, respiration for com-

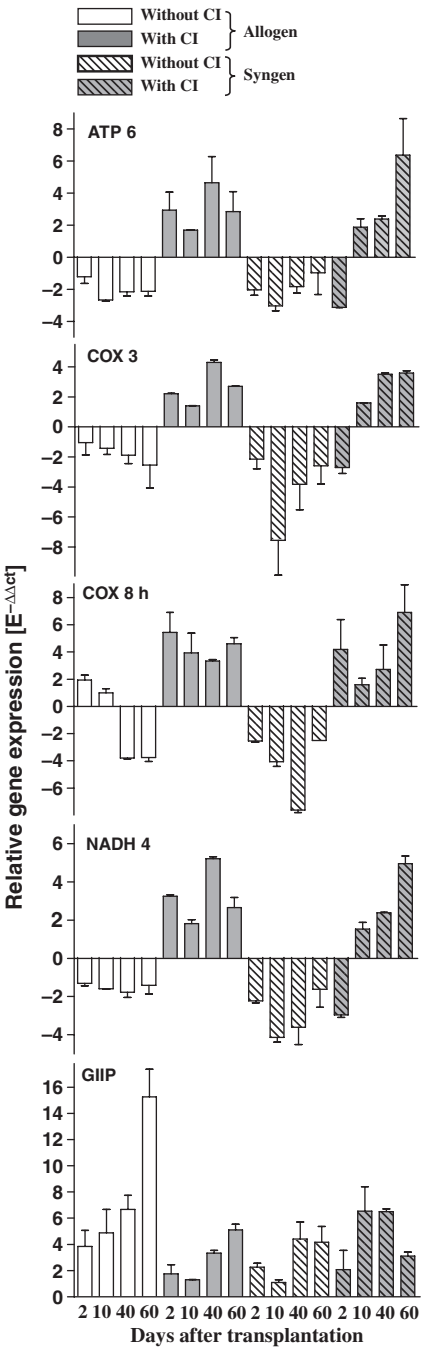

Figure 4 Real time PCR analysis of four mitochondrial genes. ATP6, COX3, COX 8h, NADH4, and one cytoplasmic gene (GIIP) were analyzed in RNA extracted from transplanted hearts. Three to five transplants were analyzed individually and the relative expression is shown as mean ± SD. Expression was read relative to gene expression from seven naïve rat hearts.

plexes I, II, and IV was significantly lower in CR-CI than in CR groups and was not restored by the addition of cytochrome *c* (Fig. 5a–d). Moreover, mitochondrial function in CR-CI, but not in CR was significantly different from corresponding syngeneic controls at this time point. Consistently, specific activities of mitochondrial marker enzymes such as mitochondrial respiratory complexes I (NADH oxidoreductase) and IV (COX), as well as mitochondrial matrix enzyme citrate synthase (CS) were unchanged in syngeneic controls, but decreased significantly in both CR and CR-CI as measured by spectrophotometry (Fig. 5e–h). Similar to the effects of CI on mitochondrial respiration (Fig. 4), the decline in the enzymatic parameters was faster and more evident in CR-CI when compared with CR (Fig. 5e–g). At day 10, activities of COX and CS were significantly diminished in CR-CI but not in CR when compared with baseline controls ($P < 0.01$ and $P > 0.05$, respectively) (Fig. 5f,g). The activity of respiratory complex I dramatically declined in both CR and CR-CI and was significantly lower than in corresponding isografts at day 10 (Fig. 5e; $P < 0.01$). At day 40, CS activity differed significantly between CR and CR-CI groups (Fig. 5g). After prolonged reperfusion at day 60, the decline in mitochondrial enzyme activities was comparable in allogeneic groups with and without cold ischemia, but significantly different from syngeneic controls. Both complex IV (COX) enzymatic activity and respiration with complex IV substrates (TMRD + ascorbate) declined similarly to approximately 50% of corresponding syngeneic controls at days 60 in CR and CR-CI (Fig. 5f,c, and d). Furthermore, enzymatic complex I activity (Fig. 5e) was almost equally decreased in both CR and CR-CI groups at day 60, while respiration with complex I substrates glutamate + malate was significantly different in CR when compared with CR-CI at that time point (Fig. 5a). The activity of the cytosolic nonmitochondrial enzyme lactate dehydrogenase (LDH) was similarly decreased in CR and CR-CI, but unchanged in syngeneic control groups (Fig. 5h).

Notably, respiratory and enzymatic parameters of mitochondria correlated well with cardiac performance (Fig. 6 a–c). Activities of mitochondrial matrix marker enzyme citrate synthase (CS), mitochondrial cytochrome *c* oxidase (COX), and ADP-stimulated respiration declined in parallel with a decrease of graft function (Fig. 6) ($r^2 = 0.88$; 0.77 and 0.81 respectively). The activity of nonmitochondrial enzyme LDH correlated with cardiac score to a much lesser extend ($r^2 = 0.37$) (Fig. 6d) when compared with parameters of mitochondrial function. Furthermore, mitochondrial active complex I respiration (glutamate + malate + ADP; Fig. 6e) and COX activity (Fig. 6f) correlated well with marker of mitochondrial content citrate synthase only in allografts (open symbols; $r^2 = 0.94$ and 0.90, respectively), but not in isografts (closed symbols; $r^2 = 0.151$ and 0.036). This difference indicates that general loss of mitochondrial functional capacity is involved in the mechanisms associated with CR, in

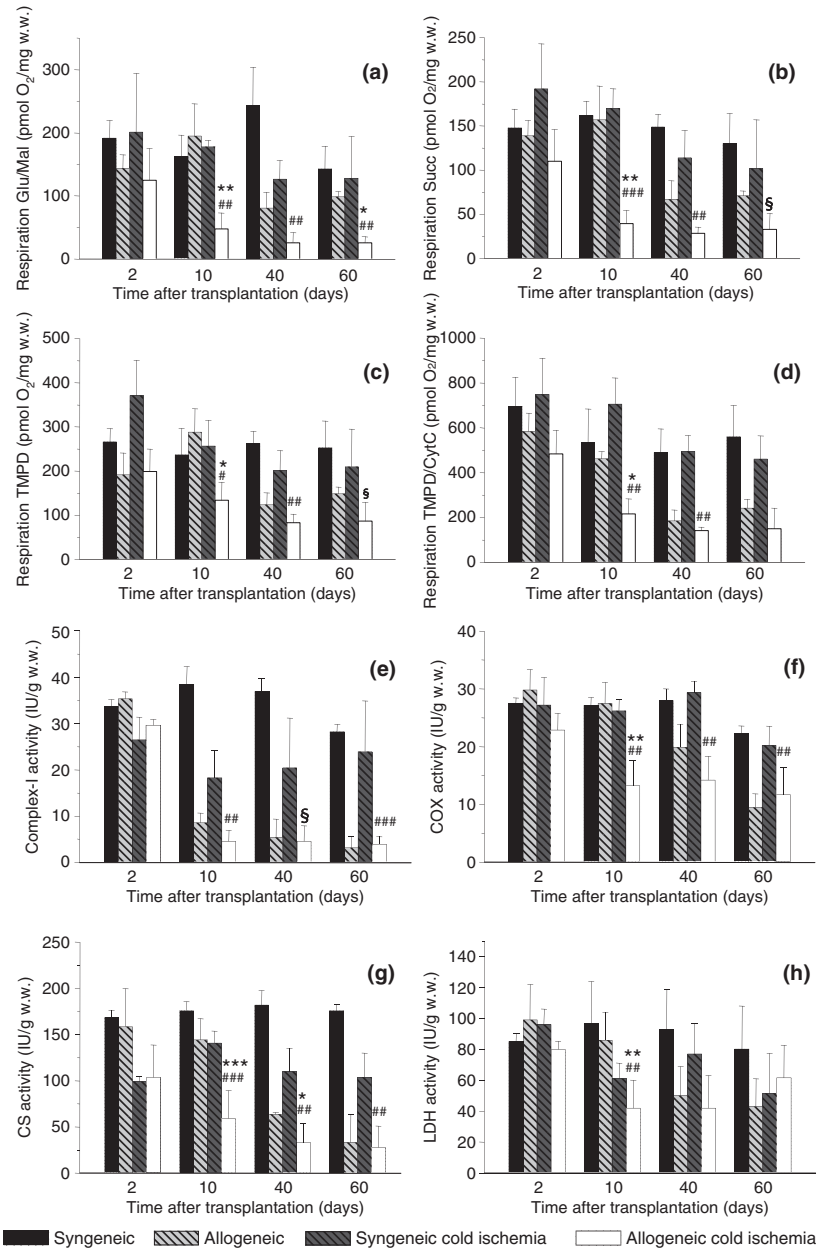

Figure 5 Mitochondrial respiration through different segments of the electron transport chain and enzymatic defects after CR and CR-CI in allogeneic and syngeneic transplants. (a) Complex I respiration (Glu/Mal; 10 mM glutamate and 5 mM malate), (b) complex II respiration (Succ; 10 mM succinate after inhibition of complex I with 0.5 μM rotenone), (c) complex IV respiration (0.5 mM TMPD and 2 mM ascorbate after inhibition of complex III with 5 μM antimycin A), (d) complex IV respiration + cytochrome *c* (0.5 mM TMPD, 2 mM ascorbate and cytochrome *c* 10 μM). In all cases, respiration was measured after the addition of 1 mM ADP (state 3 respiration). Respiration rates are expressed in pmols oxygen per second per mg wet weight. Values are mean ± SD (allogeneic CR, CR-CI: $n = 5$; syngeneic SYN, SYN-CI: $n = 3$; at each time point after transplantation). Two separate determinations for each heart. Significantly different from corresponding group without CI: $^{*}P < 0.05$; $P < 0.01$; $^{**}P < 0.01$; $^{***}P < 0.001$. Significantly different from corresponding syngeneic group: §$P < 0.05$; #$P < 0.01$; ##$P < 0.001$. (e) Activity of mitochondrial respiratory complex I (NADH:ubiquinone-1 oxidoreductase), (f) activity of mitochondrial respiratory complex IV (cytochrome *c* oxidase, COX), (g) activity of mitochondrial Krebs cycle enzyme citrate synthase (CS), (h) activity of cytosolic enzyme lactate dehydrogenase (LDH). Activities are expressed in international units per g wet weight. Values are mean ± SD (allogeneic CR, CR-CI: $n = 5$; syngeneic SYN, SYN-CI: $n = 3$; at each time point after transplantation). Two separate determinations for each heart. Enzymatic activities of muscle homogenates from naïve hearts were: CS, 167.6 ± 21; Complex I, 62.3 ± 8; COX, 30.2 ± 3; LDH, 94.6 ± 14 units per g wet weight. Significantly different from corresponding group without CI: $^{*}P < 0.05$; $P < 0.01$; $^{**}P < 0.01$; $^{***}P < 0.001$. Significantly different from corresponding syngeneic group: §$P < 0.05$; #$P < 0.01$; ##$P < 0.001$.

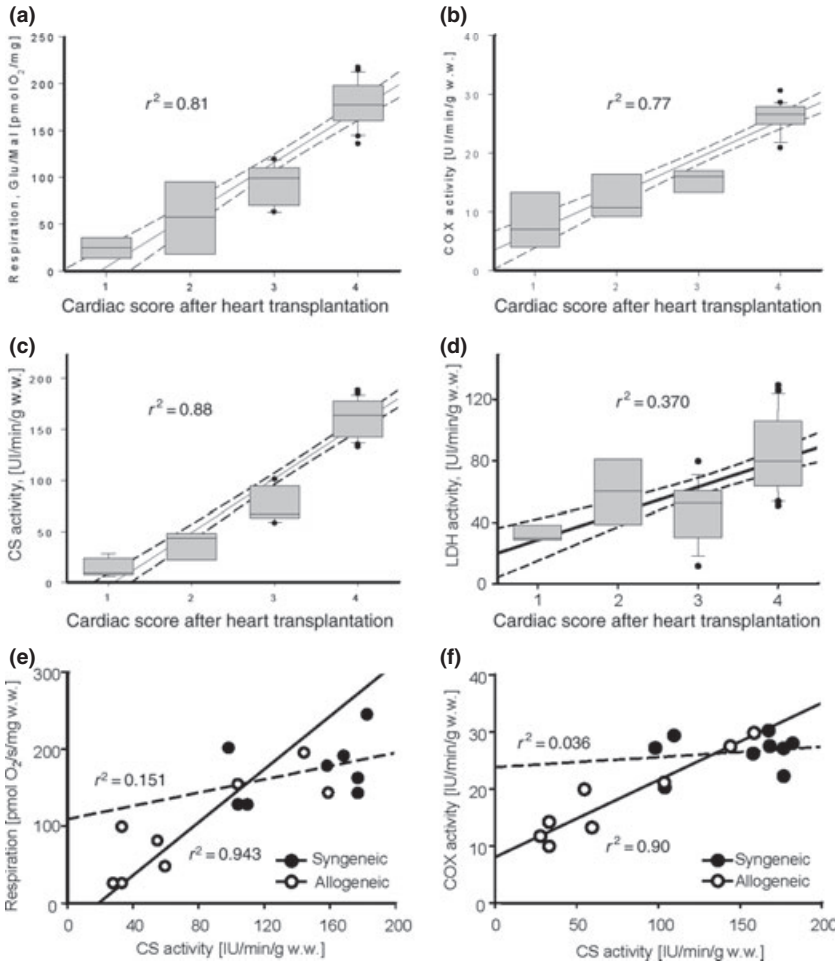

Figure 6 Mitochondrial respiration and enzymatic activities correlate with cardiac score after CR and CR-CI. Median box plots show (a) respiration with complex I substrates Glu/Mal, (b) activity of complex IV (cytochrome *c* oxidase, COX), (c) activity of citrate synthase (CS), (d) activity of lactate dehydrogenase (LDH) as a function of cardiac score. Data from all groups and time points after transplantations are combined. n = 4–10 for each score. (e and f) Mitochondrial respiration and COX activity correlate with mitochondrial marker CS in allogeneic but not in syngeneic transplants. (e) Dependence of mitochondrial ADP-stimulated complex I respiration and CS activity, (f) dependence of mitochondrial respiratory complex IV (COX) and CS activity. Allogeneic (open symbols) and syngeneic (closed symbols) groups are combined.

contrast with more specific damages of mitochondrial respiratory complexes in CI, suggesting that not a single alteration but the combination of the effects as outlined above is responsible for cold ischemia- and chronic rejection-induced mitochondrial injury.

Discussion

In this study, alterations in graft function, gene expression, graft histomorphology, and mitochondrial as well as enzymatic function during the development of rejection were investigated with particular regard to the impact of prolonged (10 h) cold ischemia. Our data indicate that mitochondria related gene expression and mitochondrial function are substantially compromised with the progression of CR and show that prolonged ischemia prior to transplantation impacts on this phenomenon.

Compared with other organs, the heart presents intrinsic difficulties for preservation over longer periods of time. Although hypothermia suppresses basal metabolic rate and modifies signaling pathways towards expression of protective genes [31–33], it does not entirely protect organs against ischemia. Tanaka *et al.* have recently described a linear correlation between ischemia time and degree of coronary artery disease in a rat cardiac transplant model, but there is ongoing controversy over the question whether ischemia *per se* is able to induce myointimal proliferation, or if an allogeneic response is a prerequisite for this event [8–12,34–37]. In our model, the antigenic stimulus had a predominant impact on the development of CR. CI

impacted on organ function early (2 and 10 days after transplantation) as well as late after transplantation (40 and 60 days). CI had a marginal effect on isografts, whereas function and histology are paralleling myointimal cell proliferation after CI in allografts (Figs 2 and 3).

Guided by the gene expression profiles (microarray), a set of mitochondrial genes representing key elements of mitochondrial oxidative phosphorylation was selected for qPCR analysis. While microarray technique was used as a screening tool and RNA from individual animals was pooled, RNA of individual animals was used for PCR analysis. CI was associated with an upregulation of mitochondria related genes, while transplantation without CI resulted in downregulation of this gene cluster (Fig. 4). At day 2, a set of mitochondrially encoded genes including genes encoding for ATPase6, COX3, and NADH4 was downregulated in syngeneic transplants exposed to CI (Fig. 4). In contrast, the nuclearly encoded COX-8H was upregulated and did not show such an expression pattern. COX3 and COX-8H are subunits of cytochrome *c* oxidase, the terminal enzyme in mitochondrial electron transport (complex IV) both essential for the activity of this complex. ATP6 is a subunit of the membrane associated F0 part of F0F1-ATP synthase relevant for proton translocation and thus ATP synthesis. NADH4 is a key component of the hydrophobic fragment of respiratory complex I. The regulation of mitochondrial genes in response to CI may be related to the effects of B7 family proteins expression [38].

In a previous trial, we have elucidated the multiple mechanisms resulting in mitochondrial injury in a syngeneic model of prolonged cold ischemia-reperfusion, including specific defects in respiratory complexes, mitochondrial uncoupling, and cytochrome *c* release [14,15,20,21]. Defective mitochondria are considered an important source (as well as a target) for overproduction of reactive oxygen species (ROS), inflicting additional damage to mitochondria (an amplification effect) and contributing to ischemia/reperfusion injury [39]. However, the increase in ROS might also be the cause and not the only result of a compromised mitochondrial respiration in CR in this study.

Rates of NADH oxidation and mitochondrial oxygen consumption were significantly decreased in allografts when compared with isografts (Fig. 5). Moreover, respiration and COX activity correlated well with mitochondrial marker enzyme CS in allogenic, but not in syngenic groups (Fig. 6e and f). This indicates that progression of chronic rejection is associated with general loss of mitochondria rather than with specific defect of particular respiratory complex.

It is interesting to note that ischemia caused a decrease in graft function as early as day 10 after transplantation in allografts (Fig. 2). Histomorphological changes at that time point were marginal, and we therefore speculate that the impaired mitochondrial function is affecting graft performance at this early time point. The increase in myointimal proliferation paralleled the deterioration of mitochondrial function in allografts transplanted without ischemia. This further underlines that the degree of myointimal proliferation represents the consequence of the sum of the individual injuries to the graft rather than an initiated and then self-maintained mechanism as suggested by others [40,41]. Monitoring mitochondrial function might therefore be a valuable tool to detect progression of CR and potentially help to predict the risk of myocardial dysfunction or sudden death.

Extrapolating from findings in a kidney transplant model, cold ischemia, and reperfusion together with allogenicity may also increase inflammation and cellular infiltration early and late after transplantation thereby enhancing vascular damage [42]. While proof of a cause-effect relation would require an investigational trial, we hypothesize that myocardial ischemia and hypoxia caused by both the I/R injury (early after transplantation) and myointimal proliferation with luminal narrowing impact on blood flow resulting in an alteration of mitochondrial gene and protein expression and mitochondrial function in cardiomyocytes. In a study by Tung *et al.*, expression of myocardial Bcl-2 and Bax was found increased in CR and apoptosis as well as Bcl-2/Bax ratio correlated with graft survival in CR, underlining that alterations in the expression of mitochondrial apoptosis regulatory proteins impact on long-term heart allograft survival [13].

Our group has previously analyzed the activity patterns of mitogen-activated protein kinases (MAPKs) ERK, JNK, and p38 during IR and probed into their role in the perturbation of mitochondrial ROS and Ca^{2+} homeostasis and cardiomyocyte death. A strong activation of MAPKs and a rise in mitochondrial ROS and Ca^{2+} during early reoxygenation were found. Inhibition of p38 kinase efficiently prevented ROS production, Ca^{2+} overload and cell death, indicating that IR injury triggers mitochondrial damage and Ca^{2+} overload through MAPKs activation [43].

Cold ischemia, transplantation, and chronic rejection are a complex cascade of events with multiple molecular mechanisms involved. In summary, we believe that I/R causes inflammation and aggravates the alloimune response in addition to microstructural and functional alterations of mitochondria. Progression of chronic rejection resulting in ischemia and hypoxia of the myocardium further aggravates mitochondrial damage and alterations of enzymatic activity.

We have identified a close association among CR, CI, and the loss of mitochondrial respiratory function and enzymatic activities. These findings may provide a basis for developing a sensitive test for the early detection of CR. Our findings suggest that immunological and

nonimmunological mechanisms contribute to mitochondrial injury in CI and CR. Data derived from this work, however, should be read and interpreted in the light of the limitations of the chosen model. Hearts in the rat model are transplanted heterotopically, and ventricles remain functionally unchallenged, which may impact on metabolism and energy consumption.

Taken together, the investigation of cardiac contractile function, gene expression, mitochondrial respiration, and enzyme activities in a rat model for CR indicate that prolonged cold ischemia substantially contributes to the progression of CR in the heart. A significant reduction in mitochondrial respiratory capacity in response to CI and CR suggests an important role of these organelles in the loss of organ function. Interventional studies are warranted to prove the relevance of mitochondria in ventricular arrhythmia, sudden cardiac death or heart failure during chronic rejection after cardiac transplantation.

Authorship

SS: participated in research design, overseeing the execution of experiments, data analysis, and writing the article. AA: participated in research design, microarray and RT-PCR data analysis, and writing of the article. JH: participated in performance and data analysis of microarray and RT-PCR. TH: participated in data analysis of histology and writing of the article. OR and HM: participated in performance of rat cardiac transplantations. PO: participated in performance of histology. GB and RM: participated in research design and writing of the article. WM and JT: participated in research design and data analysis. DS: participated in performance of respirometry and enzyme activity. AVK: participated in performance and data analysis of respirometry and enzyme activity and writing of the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gene expression early after transplantation.
Table S2. Gene expression late after transplantation.
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