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ORIGINAL ARTICLE

CORMs protect endothelial cells during cold preservation, resulting in inhibition of intimal hyperplasia after aorta transplantation in rats

Hui Song,[1,2] Simone Hoeger,[1] Jan-Luuk Hillebrands,[3] Isabell Mandel,[1] Ralf Loesel,[4] Grietje Beck,[5] Lothar Schilling,[6] Peter Schnuelle[1] and Benito Yard[1]

1 V[th] Medical Clinic, University Hospital Mannheim, University of Heidelberg, Mannheim, Germany
2 School of Dentistry, Shangdong University, Jinan, China
3 Department of Pathology & Laboratory Medicine, Division of Pathology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands
4 Georg Simon OHM, University of Applied Sciences Nuernberg, Prinzregentenufer Nuernberg, Germany
5 Department of Anesthesiology, University Hospital Mannheim, University of Heidelberg, Mannheim, Germany
6 Division of Neurosurgical Research, Medical Faculty, Mannheim, Germany

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Correspondence
Dr. Simone Hoeger, V. Medizinische Klinik, Klinikum Mannheim, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany.
Tel.: +496213300331; fax: +496213300333; e-mail: simone.hoeger@medma.uni-heidelberg.de

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Summary

Allograft vasculopathy is the leading cause for chronic transplant loss. We investigated if the addition of carbon monoxide releasing molecules (CORMs) to the preservation solution would protect the endothelium from cold preservation injury in an aortic transplantation model. In particular, we tested if CORM preserve vascular functioning and limit neo-intima formation following cold preservation (Cp). Abdominal aortas from Lewis or Fisher rats were subjected to Cp in University of Wisconsin (UW) solution to which 50 μM of CORM-3 was added or not. Hereafter, whole mount staining, acetylcholine mediated vasorelaxation (AMV) and aortic transplantation was performed. *In vitro* CORM-3 protected human umbilical vein endothelial cells from Cp injury and prevented denudation and intercellular gap formation in aortic grafts. Cp resulted in loss of AMV of aorta segments. By contrast, AMV was preserved after the addition of CORM-3 during Cp. Two months after transplantation Cp of aorta grafts resulted in an increased adventitial remodelling and neo-intima formation. This was significantly blunted by CORM-3 in syngeneic recipients. Our study demonstrates that addition of CORM-3 to UW solution prevents endothelial damage, thereby maintaining vascular function directly after cold preservation. Hence, our findings might offer a novel strategy to prevent vascular damage during CP.

Introduction

Static cold storage is widely used for preserving graft function prior to transplantation. However, organ quality and function are deteriorating with increasing cold ischemic time. During cold preservation, a series of events occur, such as oxidative stress, intracellular iron release and calcium overload [1–3]. Cold preservation is a major cause of pretranplantation injury of allografts and is significantly associated with initial nonfunction and late graft failure [4–7].

Chronic transplant vasculopathy remains a leading cause for chronic allograft dysfunction [8,9] and is characterized by prominent vascular remodelling in which the luminal areas of arteries become obliterated due to occlusive intimal hyperplasia. Although the etiology of intimal hyperplasia is not well understood, pretransplantation injury is recognized as an important cause for this

complication. In addition, as donor allogeneic endothelial cells of the graft's vasculature are under continuous attack by the recipient's immune system, an imbalance between damage and repair might contribute to intimal hyperplasia [10]. Migration of smooth muscle cells and proliferation of recruited progenitor cells in neo-intima formation have been identified to play a crucial role in experimental models of transplant vasculopathy [11,12].

Carbon monoxide (CO), an endogenous by-product of heme, catalyzed by heme oxygenase (HO), mediates several cell biological functions, including cGMP-mediated vasorelaxation [13,14], inhibition of cell proliferation [15,16], inhibition of apoptosis [17] and suppression of inflammation [18]. Based on these effects, it is not surprising that CO has the propensity to protect different organs from ischemia and reperfusion injury [19–23]. It has been postulated that the mechanism by which CO mediates organ protection is related to HO-1 induction and activation of the p38 MAPK signaling pathway [13,17,18]. In most organ transplantation studies involving CO, its delivery was mediated through ventilation [19,21,22]. As a novel approach, Motterlini *et al.* have reported on a new class of compounds, termed CO-releasing molecules (CORMs), which are able to release CO in a controllable manner under physiological conditions [24]. In particular, fully water-soluble CORM-3 [tricarbonylchloro(glyconato)-ruthenium(II)] and CORM-A1 (sodium boranocarbonate), can potentially be used as additives in preservation solutions [23,25].

Endothelial cell injury is a prominent feature of cold ischemia [26]. Therefore, strategies to mitigate the deleterious effect of cold preservation might be a promising approach to limit intimal hyperplasia. In the present study we employed *in vitro* and *in vivo* models to test the hypothesis that the addition of CORM-3 to the preservation solution protects vascular endothelial cells from damage and hence better preserves vascular function after cold storage. In addition, we hypothesize that a reduction in endothelial cell damage will significantly decrease neo-intima formation. To study the effect of cold preservation as a single cause for neo-intima formation a syngeneic Lewis-to-Lewis aortic transplant model was used. Moreover, it was tested if CORM-3 was able to inhibit neo-intima formation in the allogeneic Fisher-to-Lewis model.

Materials and methods

Drugs and reagents

Endothelial cell growth medium MedKIT, Phenol-red free medium (Promocell, Heidelberg, Germany), PBS (Invitrogen, Karlsruhe, Germany), Foetal bovine serum (FBS) Gold (PAA laboratories GmbH, Pasching, Austria), Trypsin/EDTA solution, DMSO, TritonX-100, tricarbonyldichlororuthenium(II) dimer $\{[Ru(CO)_3Cl_2]_2\}$, glycine, sodium ethoxide, tetrahydrofuran, acetylcholine (Ach), sodium nitroprusside (SNP), 1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) (Sigma, St Louis, MO, USA), Bovine Serum Albumin (SERVA, Heidelberg, Germany), 37% formaldehyde (Mallinckrodt Baker, Deventer, The Netherlands).

Synthesis of tricarbonylchloro(glycinato)ruthenium(II)

Tricarbonylchloro(glycinato)ruthenium(II) $\{[Ru(CO)_3Cl(glycinate)]\}$ was synthesized from a commercially available compound, tricarbonyldichlororuthenium(II) dimer $\{[Ru(CO)_3Cl_2]_2\}$ as previously described [27]. Briefly, $[Ru(CO)_3Cl_2]_2$ (0.5 g) and glycine (0.151 g) were placed under nitrogen in a round-bottomed flask. Methanol (291 ml) and sodium ethoxide (0.132 g) were added and the reaction was allowed to continue under stirring for 18 h at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in tetrahydrofuran (THF). The yellow solution was evaporated down to give a pale yellow solid (yield 92–96%) and was aliquoted and stored in closed vials at room temperature. For each experiment CORM-3 was dissolved freshly in PBS.

Cell culture and CORM-3 treatment

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords and cultured as described previously [2]. The cells were seeded in 24-well plates in basal endothelial medium supplemented with 10% FBS and essential growth factors until they formed a confluent monolayer. Cells were then stored for 24 h at 4 °C in UW solution in the presence or absence of different concentrations CORM-3. In some experiments cells were pretreated for 6 h with different concentrations of CORM-3 prior to cold preservation. Thereafter the cells were washed two times with PBS and stored for 24 h at 4 °C in UW solution. For the experiments in which ODQ was used, HUVECs were pretreated for 1 h with 50 μM of ODQ or left untreated. ODQ was also present during cold preservation in the pretreated group. Degassed CORM-3 was produced by dissolving CORM-3 in water and putting the solution for 24 h in a vacuum device. Directly after cold preservation cell damage was assessed by measuring lactate dehydrogenase (LDH) (Roche Diagnostics, Mannheim, Germany) activity in the supernatants as recommended by the manufacturer. Only HUVECs from passage 2 to 6 were used in this study. A total of five experiments were performed.

Animals

Inbred male Fisher and Lewis rats weighing 250–300 g were obtained from Charles River (Sulzfeld, Germany). Animals were kept under standard conditions and fed standard rodent chow and water *ad libitum*. All procedures were performed according to the 'Guide for the Care and Use of Laboratory Animals' published by the National Academy of Sciences and were approved by the local authorities (RP Karlsruhe, AZ 35–9185.81/91/07).

Vascular function

Abdominal aortas were explanted from Lewis rats and either directly used for subsequent experiments or stored for 24 h at 4 °C in UW solution in the presence or absence of 50 μM CORM-3. This concentration was chosen based on the study of Musameh *et al.* [25]. Vascular function measurement was performed as previously described [28]. Briefly, the aortas were prepared and cleaned from periadventitial fat and surrounding connective tissues, cut transversely into 2-mm width rings and mounted on stainless steel hooks in individual organ baths containing Krebs–Henseleit solution (NaCl 119 mM, KCl 3 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.5 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 20 mM, Glucose 10 mM) at 37 °C and aerated with 75% N_2, 20% O_2 and 5% CO_2. The aortic rings were placed under a resting tension of 15 mN and equilibrated for 60 min. During this period, tension was periodically adjusted to the desired level and the Krebs-Henseleit solution was changed regularly. The aortic rings were challenged by increasing concentration of KCl (20 mM, 30 mM, 40 mM, 50 mM, 80 mM and 124 mM) in Krebs solution in which NaCl was substituted by KCl to keep osmolarity constant. The maximal contraction forces to potassium chloride were determined according to the responsive curves. Aortic rings were washed until resting tension was obtained again. KCl 50mM was used to precontract the aortic rings until a stable plateau was reached. Concentration response curves were constructed by cumulatively adding acetylcholine (Ach, 10^{-9}–10^{-4} M) or sodium nitroprusside (SNP, 10^{-9}–10^{-5} M) to elicit the endothelium-dependent (Ach) or endothelium-independent (SNP) relaxations. The data were monitored and analyzed using the LabView-based software previously developed in our lab.

Whole mount aorta staining

To study the effect of cold preservation with or without CORM-3 on endothelial integrity, freshly isolated or cold preserved aortas were transversely cut into 3–4 mm segments and fixed at room temperature for 2 h in freshly prepared 4% formaldehyde. After fixation, the segments were washed three times with PBS for 1 h. Subsequently, the segments were incubated for 20 min in PBS containing 1% BSA, 0.5% Triton and 5% rabbit serum, followed by extensively washing with PBS. The segments were incubated overnight at 4 °C with a purified mouse anti-rat CD31 antibody (BD Pharmingen, Heidelberg, Germany) diluted in PBS. After washing, rabbit anti-mouse Texas-red conjugated IgG was added for 2 h. Nuclei were visualized by addition of TOTO3 10 min before mounting. Finally, the aorta segments were longitudinally cut into halves and mounted onto glass slides in 50% glycerol/PBS, with the luminal side up. Fluorescence was analyzed by confocal microscopy using the corresponding excitation and emission wavelengths for Texas red (596/620 nm) and TOTO3 (642/660 nm).

Aorta transplantation

Aorta transplantation was performed in the syngeneic Lewis-to-Lewis and allogenic Fisher-to-Lewis strain combinations. Both in the syngeneic and in the allogeneic model a total of six animals were transplanted in the CORM-3 and control group. All animals received ketamine and xylazine (100 and 6 mg/kg i.p., respectively) as an anesthetic agent. A 1.5 cm long segment of the descending abdominal aorta was removed, thoroughly flushed with UW-solution and either directly transplanted or subjected to 24 h of cold preservation in UW solution. Aortas were preserved in UW solution at 4 °C either in the presence or absence of 50 μM of CORM-3. Aortas were orthotopically transplanted in between the renal arteries and bifurcation. The cranial anastomosis was made as close to the renal arteries as technically possible to minimize diameter difference. End-to-end anastomoses were performed using 9-0 nylon suture. Total warm ischemic time in the recipient was consistently ~30 min. Grafts were harvested 2 months after transplantation and then processed for histological analysis as described below. All animals survived 2 months after transplantation.

Quantification of intimal hyperplasia

Two months after transplantation, aorta grafts from each groups ($n = 5$) were harvested, fixed in 4% formaldehyde and mounted in paraffin. Sections were cut (5 μm) from at least four different segments of the graft, deparaffinized according to standard methods and stained with haematoxylin/eosin.

The degree of intimal hyperplasia was assessed by morphometric analysis and expressed as mean ratio of the intima surface (μm^2)/intima + media surface (μm^2) in different sections and segments of the transplanted aorta.

Statistical analysis

Numerical data are presented as mean ± SD or mean ± SEM. Statistical analysis for all studies involving cultured endothelial cells and vascular function was performed using the Kruskal–Wallis test with option for multiple comparisons (StatsDirect 2.2.8, Aswell, UK). Statistics for cell culture comprises a total of five independent experiments, each condition tested in triplicate. Statistics for vascular function comprises a total of five animals per group, each group consisting of at least 15 aorta segments. For analysis of light microscopy, Fisher's exact test was applied, comprising six animals per group and for each graft at least five sections. A P value of less than 0.05 was considered as significant.

Results

CORM-3 protects endothelial cells against cold preservation damage

Addition of CORM-3 to the preservation solution protected endothelial cells against cold preservation damage in all HUVEC cell lines tested ($n = 5$). The protective effect of CORM-3 was detected over a wide concentration range of CORM-3, with maximal inhibition already occurring at 16 μM (Fig. 1a, black circles). To test if the protective effect of CORM-3 was dependent on the release of CO, similar concentrations of degassed CORM-3 were tested in parallel experiments. In contrast to CORM-3, degassed CORM-3 did not inhibit LDH release during cold preservation (CORM-3 versus degassed CORM3, $P < 0.01$) (Fig. 1a, filled squares). We also investigated if CORM-3 pretreatment of HUVEC, followed by cold preservation in the absence of CORM-3, was equally effective as addition of CORM-3 only during cold preservation. Pretreatment was less effective compared to treatment during cold preservation, as significant higher concentrations of CORM-3 were required for protection (Fig. 1a, open triangles). None of the concentrations of CORM-3 used in this study were toxic as assessed by trypan blue exclusion. Cell viability was for each concentration above 95% (data not shown). The protective effect of CORM-3 was largely independent of cGMP, since inhibition of guanylate cyclase (GC) by ODQ only marginally, though significantly, inhibited the protective effect of CORM-3 on cold preservation-induced endothelial damage (Fig. 1b). Similar results were observed with higher concentrations of ODQ (data not shown).

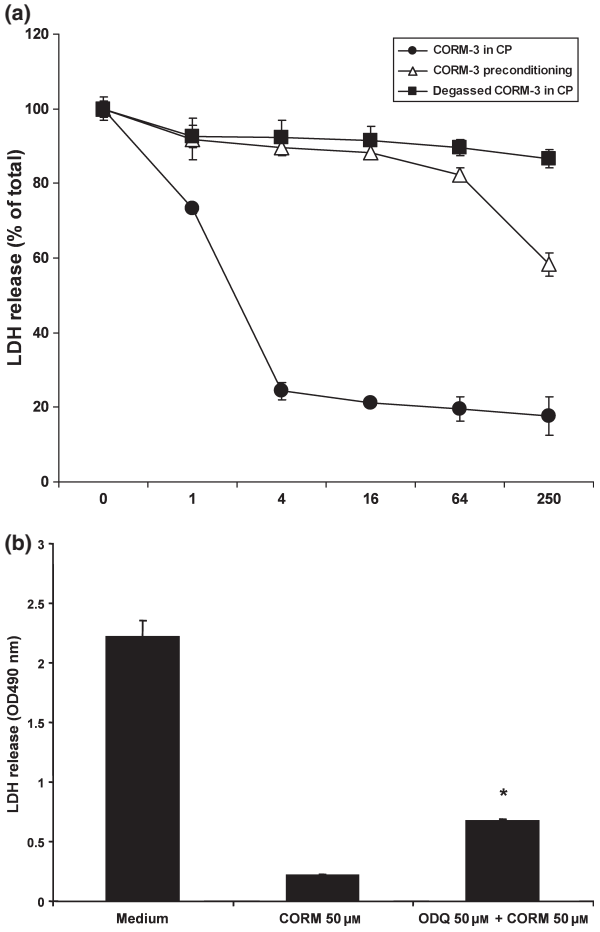

Figure 1 CORM-3 treatment renders vascular endothelial cells resistance to hypothermia-induced cell damage. (a) Different concentrations of CORM-3 were either added to HUVECs during cold preservation (filled circles) or HUVECs were pretreated for 6 h with similar concentrations of CORM-3 before cold preservation was started (open triangles). A degassed solution of CORM-3 (filled squares) tested in the same concentration range was used to demonstrate that the effect was mediated by CO release. The results are expressed as percentage of total LDH present in an equal number of cells not subjected to cold preservation (*$P < 0.01$, versus no addition of CORM during cp). (b) HUVECs were pretreated for 1 h with 50 μM of ODQ or left untreated. Hereafter the cells were subjected to 24 h of cold preservation in the presence or absence of 50 μM of CORM-3. ODQ was also present during cold preservation in the pretreated group. Directly after cold storage cell damage was assessed by LDH-release in the supernatants. All experimental conditions were performed in triplicate. The results of a representative experiment are expressed as mean ± sd. A total of five experiments were performed (*$P < 0.01$, CORM versus ODQ + CORM).

CORM-3 maintains vascular function after cold preservation *ex vivo*

We next assessed if also the endothelium in aorta segments were damaged during cold preservation and to what extent this was prevented by the addition of CORM-3 to the preservation solution. The endothelium in aorta segments that were stored for 24 h at 4 °C was dramatically changed compared with freshly isolated, not cold preserved aorta's. After cold preservation, endothelial cells

displayed an irregular morphology with large intercellular gaps between the cells and in the majority of cells nuclear condensation occurred (Fig. 2, left panel). In contrast, when CORM-3 was added during cold preservation cell morphology, CD31 expression and nuclear appearance (Fig. 2, middle panel) was similar to that observed in freshly isolated aorta's that were not subjected to cold preservation (Fig. 2, right panel). Since endothelial NO production plays a critical role in vasorelaxation, we subsequently tested if this response was impaired by cold preservation. Endothelial dysfunction was defined as a reduction in maximal relaxation in response to acetylcholine. After 24 h of cold preservation, vascular function was markedly impaired, as indicated by a significant reduction in maximal relaxation compared to freshly isolated aortas (aortas with cold preservation versus freshly isolated aortas: 24.6 ± 10.7% vs. 54.1 ± 2.6%, $P < 0.01$). When CORM-3 (50 μM) was added during cold preservation, endothelial function was significantly improved, reaching a maximal relaxation of 43.4 ± 7.6% (cold preservation-CORM-3 versus cold preservation, $P < 0.05$) (Fig. 3a). In contrast to the endothelium-dependent vasorelaxation response, the endothelium-independent response was not affected by cold preservation. Addition of the NO donor sodium nitroprusside (SNP) to the organ bath resulted in a dose-dependent vasorelaxation. No significant differences between the two treatment groups and freshly isolated aorta's were observed (Fig. 3b). This indicates that endothelial cell function but not smooth muscle cell function, is impaired after 24 h of cold preservation.

CORM-3 suppresses intimal hyperplasia after transplantation

To demonstrate the influence of cold preservation on vascular remodelling, we performed abdominal aorta transplantations in the syngeneic Lewis-to-Lewis rat strain combination. Before transplantation, donor aorta grafts were stored for 24 h at 4 °C in UW solution with or without 50 μM CORM-3. When the aortas were harvested 2 months after transplantation, macroscopic inspection consistently revealed massive amounts of fibrous tissue surrounding transplants that were preserved at 4 °C in the absence of CORM-3 in all animals (Fig. 4 a, middle panel). In contrast, none of the animals that received a graft that was preserved in the presence of CORM-3 displayed any signs of adventitial fibrous remodeling (Fig. 4a, right panel). These grafts had a macroscopic appearance similar to that of aortas from naive not transplanted animals (Fig. 4a, left panel). Histological analysis revealed that 2 months after transplantation neo-intima formation was marginal in the Lewis-to-Lewis model when no cold preservation was performed (Fig. 4 b, picture to the left). Cold preservation significantly aggravated neo-intima formation when the aortas were preserved at 4 °C in the absence of CORM-3 (Fig. 4b, middle panel), while addition of CORM-3 to the preservation solution obviously attenuated neo-intima formation (Fig. 4b, right panel).

Quantitative analyses using computerized morphometry (i.e. determination of the ratio of intima surface/media + intimal surface) in different sections and segments of the graft revealed that the degree of intimal hyperplasia was significantly reduced in CORM-3-treated aorta grafts (cold preservation-CORM-3 versus cold preservation, 0.12 ± 0.01 vs. 0.45 ± 0.04, $P < 0.01$) (Fig. 4c).

To study if CORM-3 also had a beneficial effect on intimal hyperplasia in an allogeneic donor recipient combination, aorta transplantations were also performed in the Fischer-to-Lewis model. In this combination, intimal hyperplasia was more pronounced and was not affected by CORM-3 (data not shown).

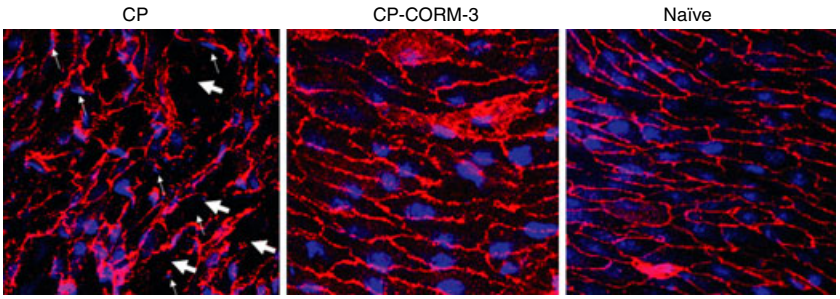

Figure 2 Influence of CORM-3 on endothelial integrity in aorta segments subjected to cold preservation. Whole mount staining for CD31 (red) was performed as described in the 'Materials and method' section. Nuclear staining is depicted in blue. Note that in the aorta segments subjected to cold preservation in the absence of CORM-3, endothelial cell morphology was irregular with intercellular gaps (bold arrows) appearing between adjacent endothelial cells. Also in the majority cells nuclear condensation (arrow) was observed (left panel). In contrast, aorta segments to which 50 μM of CORM-3 was added during cold preservation (middle panel) appeared to be similar to freshly isolated aorta segments not subjected to cold preservation (right panel). Original magnification: 200 ×.

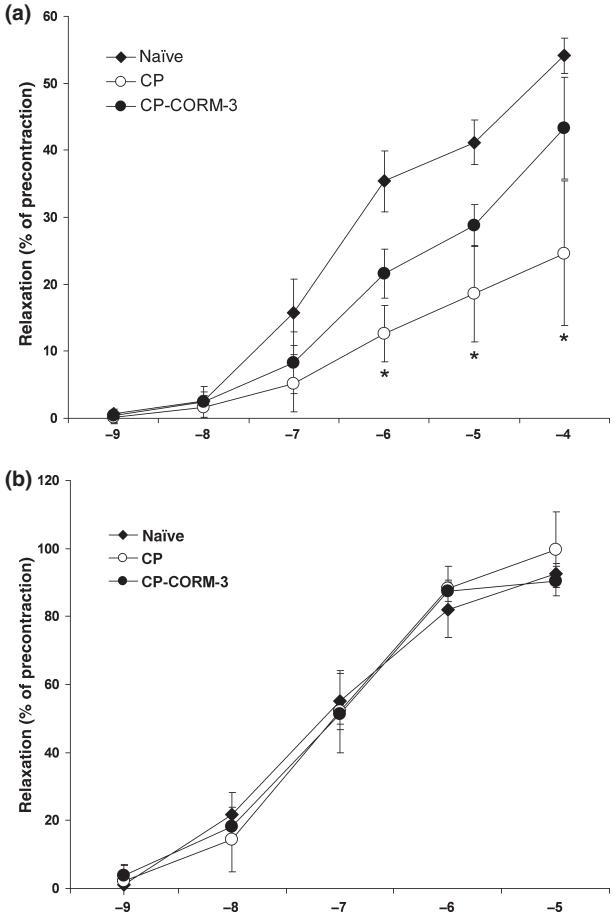

Figure 3 Administration of CORM-3 during cold preservation improves vascular function. Isolated abdominal rat aortas were subjected to cold preservation (CP) for 24 h in the presence (CP-CORM-3; black circles) or absence (CP; open circles) of 50 μM of CORM-3. Naïve aortas without cold preservation (black diamonds) were included as control. (a) Endothelial function was analyzed by endothelium-dependent relaxation in response to cumulative acetylcholine concentrations (10^{-9}–10^{-4} M). (b) Smooth muscle cell function was demonstrated by endothelium-independent relaxation in response to cumulative sodium nitroprusside concentrations (10^{-9}–10^{-5} M). Five animals were included in each group and 16–18 aortic segments of each group were analyzed. The results were expressed as mean maximal relaxation ± SEM at indicated concentration Ach or SNP (*$P < 0.05$, CP-CORM-3 versus CP).

Discussion

Chronic allograft vasculopathy (CAV) is a leading cause for late allograft loss after organ transplantation [5,6,10,11]. Both prolonged cold ischemia time and immune-mediated mechanisms are believed to contribute to CAV. A characteristic finding in CAV is intimal hyperplasia, arising from the migration and proliferation of smooth muscle cells (SMC) culminating in the formation of an occlusive neo-intima.

In the present study we show that hypothermic preservation severely affects the vascular endothelium and that this alone can lead to intimal hyperplasia after transplantation. Protection of the vascular endothelium during hypothermic preservation therefore would have a beneficial effect on intimal hyperplasia. To exclude alloimmune-mediated mechanisms in the development of intimal hyperplasia the syngeneic Lewis-to-Lewis aorta transplant model was initially employed. The allogeneic Fisher-to-Lewis model was then used to assess if CORM-3 also inhibited intimal hyperplasia in an allogeneic donor–recipient combination. The main findings of our study are the following: CORM-3 protects *in vitro* cultured endothelial cells as well as endothelial cells in situ in aorta grafts against cold preservation-induced damage. Cold preservation causes impaired endothelial function reflected by a diminished endothelium-dependent but not endothelium-independent vasorelaxation response. Addition of CORM-3 during cold preservation improves vascular function and limits intimal hyperplasia after transplantation.

CO release by CORM-3 is both temperature and pH dependent [29]. Although we did not measure the release of CO in UW solution at 4 °C, it has been reported that CORM-3 releases CO at similar temperatures within 4 h when dissolved in Celsior solution [29]. In keeping with the fact that the pH of UW solution almost equals that of Celsior solution, it is likely that a similar kinetic of CO release might be observed in the former solution.

The beneficial effect of CO on intimal hyperplasia was already documented by Otterbein *et al.* [30]. In contrast to our *in vivo* model they used an aorta transplantation model in which the recipients were treated. Both studies clearly demonstrate the usefulness of CO as potentially beneficial therapeutic option in a transplantation setting. Yet, the importance of our findings is that addition of CORM-3 to the preservation solution is effective and does not encumber the recipient.

We are however aware that a syngeneic donor–recipient combination is not reflecting the clinical practice of organ transplantation and that ongoing alloimmune-mediated vascular damage is of equal importance for the development of intimal hyperplasia. Since cold ischemia time is a risk factor for late transplant loss, this study was primarily undertaken to show that cold preservation alone can give rise to profound intimal hyperplasia, even in the absence of an allo-immune response. Hence, our study emphasizes the pivotal role of cold preservation damage on vascular remodeling and is in line with previous studies on the influence of cold preservation on vascular function and remodeling [31,32]. Our studies are also in line with a recent report by Nakao *et al.* [33] in which CO significantly attenuated I/R graft injury, reduced the

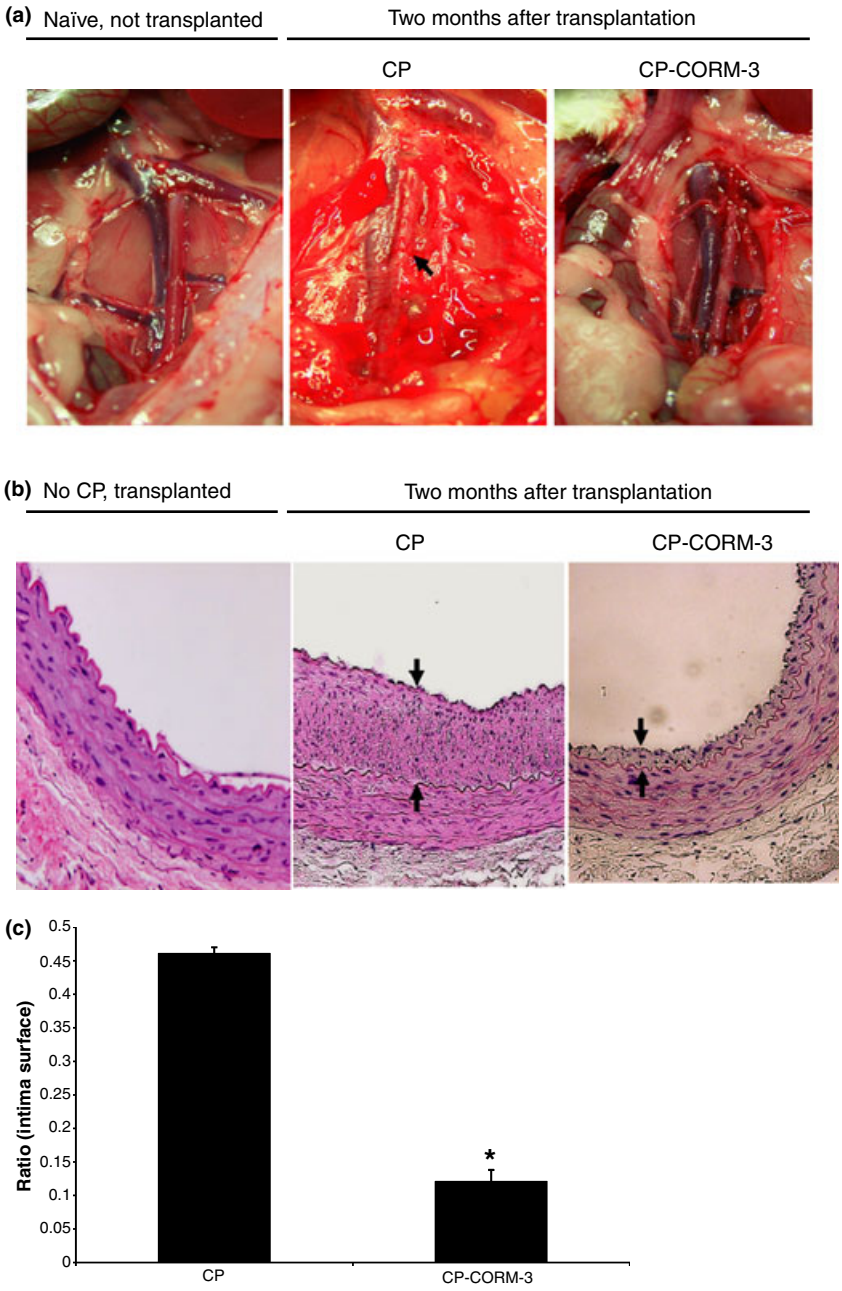

Figure 4 Addition of CORM-3 during cold preservation inhibits vascular remodeling. (a) Syngeneic aorta transplantations were performed as described in the 'Materials and methods' section. The aortas were subjected to 24 h of cold preservation in the absence (CP) or presence (CP-CORM-3) of 50 μM CORM-3. Two months after transplantation the grafts were harvested and macroscopically examined. Note the presence of fibrous tissue surrounding the graft (arrow) in the aortas subjected to cold preservation in the absence of CORM-3 (middle panel). This was not observed when 50 μM of CORM-3 was added during cold preservation (right panel). For comparison an aorta from a naïve nontransplanted rat is also depicted (left panel). (b) After macroscopic examination, the grafts were harvested and tissue sections were analyzed by light microscopy using hematoxylin & eosin staining. Representative photographs of transplanted grafts not subjected to cold preservation (No CP), subjected to cold preservation in the absence (CP) or presence of 50 μM of CORM-3 (CP-CORM-3) are depicted. Intimal hyperplasia is shown as the tissue in between the black arrows. Original magnification: 200 ×. (c) Quantitative analysis of intimal hyperplasia. Grafts from group CP and CP-CORM-3 were analyzed by means of quantitative computerized morphometry. Grafts from the No-CP group were not analyzed because intima thickening was only marginally present and only at the site of anastomosis. The degree of hyperplasia was assessed as described in the 'Materials and methods' section. The results are expressed as mean ratio [intima surface (μm^2)/intima + media surface (μm^2)] ± SEM of five different sections for each group and with $n = 6$ animals in each group (*$P < 0.01$).

infarcted area and decreased serum troponin I and creatine phosphokinase (CPK) levels after syngeneic heterotopic heart transplantation in rats.

Endothelial cell damage as a consequence of hypothermic preservation is believed to occur through a sequel of iron release, glutathione depletion and calcium accumulation in mitochondria [34]. Recently we also have shown that CORM-3 displays anti-inflammatory effects when endothelial cells are stimulated with TNF-α [35]. It therefore remains to be elucidated if the beneficial effect of CORM-3 on neo-intima formation is due to its anti-inflammatory properties, its cryoprotective properties or to both. Because we have previously shown that the anti-inflammatory properties of CORM-3 require high concentration of CORM-3, i.e. at least 500 μM [35], it seems unlikely that 50 μM of CORM-3 used in the preservation solution would influence vessel inflammation. Hence, it is more likely that the protective effect of CORM-3 is mediated via prevention of endothelial damage during cold preservation. Nevertheless we cannot rule out that the anti-inflammatory effect of CORM-3 contributes to a minor extent to the beneficial effect on intimal hyperplasia.

We also tested if CORM-3 would influence neo-intima formation in an allogenic setting in which the Fischer-to-Lewis combination was used. In contrast to the syngeneic model, CORM-3 did not influence intimal hyperplasia. We have previously shown in a fully MHC-mismatched donor–recipient combination that neo-intima formation is an alloantigen driven process. This is supported by the fact that treatment with cyclosporine A completely abolished neo-intima formation [11,12,36]. Although the Fisher-to-Lewis combination is mismatched only for minor histocompatibility antigens, it is conceivable that a low grade anti-donor response occurs in these recipients [37,38]. Therefore initial endothelial cell injury might have been prevented by CORM-3 in the Fisher aorta graft, but an on-going low grade anti-donor immune response could continue to support neo-intima formation. It remains to be elucidated if addition of CORM-3 to the preservation solution is still able to influence neo-intima formation in cyclosporine A treated recipients receiving an MHC mismatched graft.

Several studies already have shown that application of donor CO treatment can ameliorate chronic allograft nephropathy [39] and improve islet allograft survival [40]. In all of these studies, however, cold preservation was not performed. Addition of CORM-3 to the preservation solution has also been studied in heart grafts, but they were not transplanted [25]. After *ex vivo* reperfusion of these grafts, a significant improvement in systolic and diastolic function as well as in coronary flow was found. Our own data with aorta grafts also show functional improvement after cold preservation when CORM-3 was added to the preservation solution.

In vitro pretreatment of endothelial cells with CORM-3 was to some extent protective, but much higher concentrations of CORM-3 were required. Hence, the beneficial effect of donor CO conditioning might be partly lost upon prolonged cold preservation. Therefore it would be preferable to include CO in the preservation solution also.

Vascular remodeling following cold preservation and transplantation was not only restricted to intimal hyperplasia, but also the amount of fibrous tissue surrounding the graft was clearly increased. Intimal hyperplasia and expansion of the fibrous tissue were both attenuated when cold preservation was performed in the presence of CORM-3. With respect to the fibrous tissue surrounding the graft, it must be emphasized that objective measurements were not performed in this study. Although it was observed in all of the transplanted grafts in the non CORM-3 treated group and in none in the CORM-3 treated group, we cannot exclude that surgical complications were underlying this phenomenon.

In conclusion, our study demonstrates that endothelial damage during cold preservation is an eligible condition for intimal hyperplasia after transplantation and that it can occur independently of an anti-donor immune response. Prevention of vascular injury before organ implantation is of utmost importance in this regard. This objective can simply be achieved by addition of CORM-3 to the preservation solution. Nonetheless further studies are warranted to assess the general applicability of CORM-3 for solid organ transplantation in allogeneic donor recipient combinations.

Authorship

HS and SH: contributed equally to this study, performed research, analyzed data and wrote the paper. HS, SH, J-LH and IM: performed research. RL and LS: involved in the design of the research. GB: collected data. PS, BY: designed research and was involved in writing the paper.

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