Pre-ischaemic conditioning of the pulmonary endothelium by immunotargeting of catalase via angiotensin-converting-enzyme antibodies

Kai Nowak\textsuperscript{a,1,*}, Christine Hanusch\textsuperscript{d,1}, Kathrin Nicksch\textsuperscript{a}, Roman P. Metzger\textsuperscript{c}, Grietje Beck\textsuperscript{d}, Martha M. Gebhard\textsuperscript{b}, Peter Hohenberger\textsuperscript{a}, Sergei M. Danilov\textsuperscript{e}

\textsuperscript{a}Division of Thoracic Surgery, Department of Surgery, Mannheim University Medical Center, Heidelberg University, Theodor-Kutzer-Ufer 1-3, 68135 Mannheim, Germany
\textsuperscript{b}Department of Experimental Surgery, Faculty of Medicine, Heidelberg University, Germany
\textsuperscript{c}Department of Pediatric Surgery, University of Leipzig, Germany
\textsuperscript{d}Department of Anesthesiology and Critical Care Medicine, Mannheim University Medical Center, Heidelberg University, Germany
\textsuperscript{e}Department of Anesthesiology, University of Illinois, Chicago, IL, USA

Abstract

\textbf{Background:} Alleviation of oxidative stress via targeted delivery of catalase to the pulmonary endothelium by conjugation of angiotensin-converting-enzyme (ACE) monoclonal antibodies attenuates lung injury in an \textit{in vivo} model of warm lung ischaemia and reperfusion. This study evaluates treatment of lung allografts with conjugates of anti-ACE antibody with catalase (9B9-CAT) in the setting of hypothermic preservation and reports the effect on ischaemia/reperfusion injury in this model. \textbf{Methods:} Rats were injected 1 h prior to lung harvesting with mouse immunoglobulin G (IgG) (negative controls), catalase only (CAT) or anti-ACE mAb 9B9 conjugated with catalase (9B9-CAT). Lungs were flushed with low-potassium dextran (LPD) solution, excised and stored at 4 °C for 4 and 8 h. Grafts were isolated and directly reperfused at 37 °C for up to 180 min. Peak inspiratory pressure (PIP), pulmonary arterial pressure (PAP) and lung weight were measured during reperfusion. \textbf{Results:} Cold ischaemia of 8 h significantly increased lung weight gain, PIP and PAP in non-immune mouse IgG and CAT-treated lungs than in 9B9-CAT-treated lungs ($p < 0.005$). Significantly higher catalase activity and anti-oxidative status were found in the lung tissue of animals conditioned with 9B9-CAT after 4 and 8 h of cold storage than in animals treated with catalase (CAT) alone or in animals treated with non-immune mouse IgG ($p < 0.01$).

\textbf{Conclusion:} These results validate immunotargeting by anti-ACE mAb conjugated with catalase as a prospective and specific strategy to augment anti-oxidative defence of the pulmonary endothelium during lung transplantation. Vascular immunotargeting of anti-oxidative enzymes could limit reactive oxygen species mediated ischaemia—reperfusion (I/R) injury of the lung and has the potential to become a promising modality for extension of the viability of banked transplantation tissue.

\section{1. Introduction}

Lung transplantation is used to treat a variety of end-stage pulmonary diseases. Cold preservation and ischaemia—reperfusion (I/R) injury in lung transplantation has been described to be associated with enhanced risk for acute rejection and may lead to graft dysfunction in the long term [1,2]. As cold preservation is a promising modality for extension of the viability of banked transplantation tissue, we tested whether treatment of lung allografts with conjugates of angiotensin-converting-enzyme (ACE) antibody with catalase (9B9-CAT) attenuates lung injury associated with hypothermic preservation.

Deterioration in organ quality and function during cold ischaemia is mediated significantly by oxidative stress during reperfusion [2]. This injury to the transplanted lung with loss of endothelial barrier function ultimately leads to organ dysfunction and clinical complications that are associated with prolonged cold ischaemic time [3]. During lung I/R and cold preservation, reactive oxygen species (ROS) augment I/R injury [4]. Anti-oxidant enzymes, such as catalase and superoxide dismutase, are potent alleviators of oxidative stress via inactivation of ROS, especially hydrogen peroxide (H$_2$O$_2$). The concept of immunotargeting of anti-oxidative enzymes into pulmonary endothelium has also been success-
fully shown with monoclonal antibodies to several endothelial antigens, including ACE, inter-cellular adhesion molecule (ICAM) and platelet/endothelial cell adhesion molecule (PECAM) [5–7]. The preferential expression of ACE in pulmonary capillaries makes it an optimal target for therapy directed towards the pulmonary endothelium [6,8]. ACE is a membrane-anchored glycoprotein (carboxydipeptidase) that is expressed at the luminal surface of lung endothelial cells [6]. We previously demonstrated that anti-ACE mAb 9B9 could serve as a specific carrier for delivery of therapeutic substances to the lung vasculature [6–9]. By targeting the pulmonary endothelium via ACE antibodies conjugated with the anti-oxidant enzyme catalase (CAT), we have demonstrated effective limitation of I/R injury of the lung in an in vivo rat model of warm ischaemia [9].

It remained unclear whether 9B9-CAT ameliorates reperfusion injury after cold lung preservation followed by warm reperfusion, a situation similar to clinical lung transplantation.

In the present study we examine the effects of lung endothelial immunotargeting by anti-ACE mAb 9B9 conjugated with CAT (9B9-CAT) in a rat model of cold lung ischaemia followed by isolated lung reperfusion. We demonstrated that catalase conjugated with anti-ACE mAb 9B9 effectively protects lungs endothelium from oedema induced by I/R injury after cold lung preservation and provides a promising approach for protection of grafts for lung transplantation.

2. Methods

2.1. Experimental protocol

Male Wistar rats (300 g) were used in all experiments, receiving humane care in compliance with the European Convention on Animal Care (GV Solas guidelines) and were approved by federal regulations.

The animals were anaesthetised as described previously [10]. In all the animals a 14-gauge angiocatheter was inserted into the trachea by cervical tracheotomy. They were ventilated with a small animal respirator using 95% O2/5% CO2 gas, a tidal volume of 2 ml and a rate of 60 breaths/min and pulmonary inspiratory pressure (PIP) were measured at least six animals. Mean pulmonary arterial pressure (PAP) and PIP and PAP increases of more than 21 mmHg as endpoints [10]. In all the animals a 14-gauge angiocatheter was inserted into the trachea by cervical tracheotomy. They were ventilated with a small animal respirator using 95% O2/5% CO2 gas, a tidal volume of 2 ml and a rate of 60 breaths/min and PAP increases of more than 21 mmHg as endpoints [10]. Isolated lung perfusion was stopped when all of these endpoints were reached, shown in Fig. 1 as percentage of physiologic perfusion.

2.2. Physiological lung injury

To investigate the influence of lung pre-treatment before CP on PAP, PIP and oedema formation, lungs from IgG, 9B9-CAT and CAT only treated rats were stored in LPD solution for 4 and 8 h, and subsequently perfused for 180 min with warm Krebs–Henseleit solution. Lungs not subjected to CP were used for comparison (no CP). The Kaplan–Meyer analysis was performed using delta (Δ) weight increase of more than 4 g, and PIP and PAP increases of more than 21 mmHg as endpoints [10]. Isolated lung perfusion was stopped when all of these endpoints were reached, shown in Fig. 1 as percentage of physiologic perfusion.

2.3. Immunohistochemical detection of pulmonary targeting of 9B9-CAT conjugates

Histological evaluation was performed by a blinded investigator on the samples of right lower lung lobe by standardised H&E and PAS staining. The tissues were snap-frozen and stored in liquid nitrogen. These were sectioned at 5 μm by a cryostat microtome (CM 1850, Leica, Germany).

Four groups were evaluated. The first group served as negative controls with no prior preservation before isolated ventilation and perfusion (n = 9; no CP). In the second group CP was carried out for 4 and 8 h after intravenous treatment with mouse IgG at a dose of 300 μg 1 h before induction of ischaemia to serve as negative controls (4 h-IgG/8 h-IgG, each n = 6). Group 3 received 150 μg of biotinylated catalase intravenously before induction of ischaemia (4 h-CAT/8 h-CAT; each n = 6). Group 4 received 300 μg of anti-ACE mAb 9B9 conjugated with the same amount of catalase before induction of ischaemia intravenously (4 h-9B9-CAT/8 h-9B9-CAT; each n = 6). Subsequently, mAb 9B9 and catalase were biotinylated using NHS biotin (Calbiochem), at biotin–protein ratio of 10:1. Then a conjugate of biotinylated catalase and biotinylated mAb 9B9 was prepared by mixing with streptavidin as described [11].

2.4. Immunohistochemical detection of pulmonary targeting of 9B9-CAT conjugates

Histological evaluation was performed by a blinded investigator on the samples of right lower lung lobe by standardised H&E and PAS staining. The tissues were snap-frozen and stored in liquid nitrogen. These were sectioned at 5 μm by a cryostat microtome (CM 1850, Leica, Germany).

The animals were anaesthetised as described previously [10]. In all the animals a 14-gauge angiocatheter was inserted into the trachea by cervical tracheotomy. They were ventilated with a small animal respirator using 95% O2/5% CO2 gas, a tidal volume of 2 ml and a rate of 60 breaths/min with 2 cm H2O of positive end-expiratory pressure. Median sternotomy and thymectomy were performed to expose the heart—lung block was stored at 4°C for 4 and 8 h and subsequently reperfused at 37°C with warm Krebs–Henseleit solution. Lungs not subjected to hypothermic preservation were directly ventilated and perfused for similar time periods. Each group consisted of at least six animals. Mean pulmonary arterial pressure (PAP) and pulmonary inspiratory pressure (PIP) were measured continuously and recorded every 10 min (MCG, Hottinger—Baldwin—Messtechnik, Germany). Lung weight was recorded online to assess oedema formation.

Tissue of the left lung was frozen in liquid nitrogen and stored at –80°C or fixed in 4% (w/v) formaldehyde.
The slides were air-dried at room temperature (RT) for 12—24 h and stored at −30 °C. To detect administered conjugates in situ, immunohistochemistry was performed using antibodies directed against mouse IgGs. The mouse alkaline—phosphatase—anti-alkaline—phosphatase (AAPAP) technique was applied according to the manufacturer’s protocol (Dako Cytomation). The secondary rabbit anti-mouse IgG antibody (1:40, Dako Cytomation) was supplemented with reconstituted lyophilised rat serum (1:600, Dianova, Germany) to abolish any unspecific binding. Alkaline phosphatase substrate reaction with new fuchsin (100 μg ml⁻¹) and levamisole (400 μg ml⁻¹) was performed for 20 min at RT. Sections were counterstained with haematoxylin and mounted in gelatin.

2.4. Determination of catalase activity and anti-oxidative status in lung tissue

Lung tissue was stored at −80 °C. To prepare lung tissue lysates, the tissue samples were homogenised on ice with ice-cold phosphate-buffered solution (PBS), then centrifuged at 3000 × g for 12 min at 4 °C. The supernatants were aliquoted and stored at −80 °C for protein determination, catalase activity assessment and total anti-oxidant assay.

Catalase activity was measured by a standardised test kit (Bioxytech, Catalase 520, Oxis Research, Portland, OR, USA). The sample was incubated in a known concentration of H₂O₂. After incubation for exactly 1 min, the reaction is quenched with sodium azide. The amount of H₂O₂ remaining in the reaction mixture is then determined by the oxidative coupling reaction of 4-aminophenazon (4-aminoantipyrine, AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in the presence of H₂O₂ and catalysed by horseradish peroxidase (HRP). The resulting quinoneimine dye is measured at 520 nm (N-(4-antipyril)-3-chloro-5-sulfonate-p-benzoquinonemonoimine). Catalase activity was calculated by standard curve integration.

Total anti-oxidative status measurements were performed by a commercially available standardised colourimetric test kit (ImAnox, ImmundiagnostikAG, Bensheim, Germany). Anti-oxidative capacity is assessed by the reaction of anti-oxidants in the sample with a defined amount of exogenously provided H₂O₂. The anti-oxidants in the sample eliminate a certain amount of the provided H₂O₂. The residual H₂O₂ is determined colourimetrically by an enzymatic reaction, which involves the conversion of 3,3′,5,5′-tetramethylbenzidine (TMB) to a coloured product. After addition of a stop solution, the samples are measured at 450 nm in a microtitre plate reader. Colour corresponds to normalised controls within the test assay.

2.5. Statistical analysis

For group comparison, the measured results were quoted as mean ± SD (standard deviation). Statistical differences for catalase activity and anti-oxidative status at the end of the experiments were calculated by the Wilcoxon rank-sum test with correction by the Bonferroni method. For physiological changes in total lung weight, the Kaplan–Meier analysis was performed using delta (Δ) weight increase more than 4 g together with PIP and PAP increase as endpoints by log-rank analysis. SPSS Scientific software (SPSS Inc., Chicago, IL, USA) was used for all statistical procedures (version 20). The null hypothesis was rejected when p < 0.05.

3. Results

3.1. Effect of 9B9-CAT pre-treatment on pulmonary oedema after cold preservation

When CP time was 4 h, the increase in lung weight, PIP and PAP did not significantly differ among IgG, CAT and 9B9-CAT preserved lungs and no CP (Fig. 1A).

However, in lungs that were preserved for 8 h, lung weight increased more than 4 g within 110 min in all lungs obtained from CAT-treated rats (n = 6). 9B9-CAT pre-treatment significantly blunted the weight gain and PIP/PAP increases seen with IgG- and CAT-treated rats (Fig. 1B, no CP vs CAT, p < 0.0001; 9B9-CAT vs CAT, p < 0.01; IgG vs CAT, p < 0.005; IgG vs no CP, p < 0.01 (by log-rank test)).

3.2. Immunotargeting of catalase to the lung endothelium

In the normal rat lung, the leading cell types to express ACE are capillary endothelial cells [6]. However, a much lower content ACE is also detected in fibroblasts and in activated alveolar macrophages. The endothelium in almost the entire vasculature of the rat lung, including pulmonary arteries, pulmonary veins and the microcirculation, express ACE. By contrast, the bronchial vasculature showed a systemic-like expression pattern, that is, only 10—15% of bronchial capillaries expressed ACE [6].

To detect the uptake of 9B9-CAT conjugate, the best approach would be to trace catalase accumulation in the lung using specific anti-bovine catalase antibodies: we used bovine catalase for conjugation with mAb 9B9. However, we had not found any available anti-bovine catalase antibodies, despite a thorough search. Therefore, we tested the uptake of 9B9-CAT conjugate using antibodies to mouse IgG, in assumption that 9B9-CAT conjugate will not dissociate during experiment. In the 9B9-CAT-perfused rat lungs we detected homogeneous staining with anti-mouse IgG AP-conjugates using immunohistochemical methods. Staining was seen at all known expression sites of endothelial ACE throughout the pulmonary and bronchial circulation including arteries, arterioles, capillaries and veins after reperfusion following 4 and 8 h of CP (Fig. 2). This pattern of mAb 9B9 localisation (Fig. 2) and the physiological effect of 9B9-CAT conjugate (Fig. 1) indicates that the conjugate of anti-ACE antibodies with catalase specifically accumulates in the lung endothelial cells and exerts a physiologic effect.

3.3. Catalase activity and anti-oxidative status in lung tissue after reperfusion

Animals treated with IgG or catalase for 4 h of cold ischaemic time had lower catalase activity than those treated with anti-ACE antibody conjugated with catalase.
Cold ischaemic time negatively affects acute and long-term graft function after lung transplantation. I/R injury remains a significant cause of early morbidity and mortality after lung transplantation and has been identified as the main cause of primary graft failure. Besides injuries to the donor before and during lung retrieval, the extent of I/R injury is affected by the time of ischaemic storage, hypothermia and endothelial shear stress [2].

Several studies have addressed different non-specific approaches to improve transplant outcome by supplementation of preservation solutions with various vasoactive substances such as nitric oxide (NO), glyzerintrinitrate, prostaglandines, radical scavengers and others [2,12]. The current status of lung preservation solutions improved significantly with the introduction of LPD solutions [13], but there are large numbers of donors deemed not suitable for harvest due to pre-existing lung injury, which further complicates graft management and I/R injury. Further improvements in lung preservation and pre-treatment strategies to minimise I/R injury are essential, and could potentially improve the donor pool.

Endothelial protection and integrity during reperfusion is of critical importance to limit graft dysfunction. We hypothesised that lung endothelial cells could be preserved and protected using a specific approach via immunotargeting of ACE, which is ubiquitously expressed in lung capillaries [6]. Recently we demonstrated that lung endothelial targeting via anti-ACE antibodies conjugated with catalase (9B9-CAT) significantly decreased lung injury in a model of warm lung I/R [9]. In the present study, we tested the hypothesis that treatment of 9B9-CAT of lung allografts positively affects tissue damage associated with hypothermic preservation and reperfusion.

Our data demonstrate that 9B9-CAT pre-treatment inhibits lung weight gain and positively affects PAP and PIP, after CP and warm reperfusion. We also demonstrated that 9B9-CAT pre-treatment increases lung tissue catalase activity and total anti-oxidant capacity during reperfusion compared with catalase treatment alone.

Figure 2 shows immunohistochemical staining of native cryosections of rat lung treated with 9B9-CAT conjugate using anti-mouse IgG mAb detection (A3 and B3). A homogenous staining of 9B9-CAT conjugates was detected in all endothelial cells known to express ACE in all 9B9-CAT-treated animals during reperfusion after 4 h (A3) and 8 h of cold preservation (B3). No staining was uncovered in the investigated control groups after 4 and 8 h of cold preservation treated with mouse non-immune IgG (A1 and B1) and catalase only (CAT) (A2 and B2). Original magnification ×200, APAAP.

Extended lung storage and reperfusion has been reported to increase oxidative injury of lung grafts and are associated with DNA damage [14]. Oxidants have an important role in the initiation, augmentation and potentiation of endothelial injury [15,16]. There have been attempts to achieve protection of oxidative injury in lung transplantation by the administration of anti-oxidant enzymes [17–20]. Only Cremer et al. demonstrated a diminished pulmonary reperfusion injury in a canine model of heterotopic heart—lung transplantation by using a combination of catalase and superoxide dismutase administered during flush-perfusion and the first 20 min of reperfusion [19]. The main inability of exogenous catalase administration to provide beneficial physiologic effects lies in the specific physical location of endogenous lung catalase activity [21]. Catalase is localised to the intracellular compartment and Muzykantov et al. have shown that biotinylated catalase has a poor uptake into the intracellular compartment of the pulmonary endothelium [22].

Vascular immunotargeting of anti-oxidant enzymes to endothelial antigens (e.g., PECAM, ICAM-1 and ACE) has been
proposed to improve the therapeutic effect of these enzymes [5,7]. Recently, attenuation of in vivo lung injury has been achieved by anti-PECAM conjugates with catalase in a murine model of vascular oxidative stress and in vivo model of lung transplantation in rats [5]. Intravenously injected conjugates of PECAM and catalase accumulated in the pulmonary vasculature and retained their activity during prolonged cold storage and transplantation. Immunotargeting of catalase to donor rats augmented the anti-oxidant capacity of the pulmonary endothelium, reduced oxidative stress, ameliorated I/R injury and even prolonged the acceptable cold ischaemia period of lung grafts [5].

Despite this effective protection of lung endothelium by catalase conjugated with anti-PECAM antibodies, we believe that conjugates of anti-oxidant enzymes with anti-ACE mAbs should be much more effective and selective. In contrast to PECAM, which is significantly expressed in other organs besides the lung [6], ACE is expressed in 100% of the pulmonary capillaries compared with 10–20% of capillaries in other organs [8,23]. Conjugates of catalase with anti-ACE mAbs should offer much more specific pulmonary endothelial immunotargeting. We used anti-ACE mAb 9B9 conjugated with catalase (9B9-CAT), which have been shown to accumulate selectively in the rat lung endothelium [7,22]. It has also been shown that pulmonary uptake of 9B9-CAT is more than 100-fold stronger than that of biotinylated catalase [24]. Anti-ACE and anti-ICAM-1 conjugates with catalase have been shown earlier to limit oxidative lung injury using isolated perfused lung preparation [7]. Recently we have reported that antibody conjugates 9B9-CAT protect lungs in vivo without major side effects [9]. As warm ischaemia and reperfusion in the model used was not an exact simulation of the situation in lung transplantation, we performed organ harvesting and cold storage of lungs after preservation with LPD solution.

The mAb 9B9-CAT has been shown to provide 18-fold increase in pulmonary uptake of catalase enzyme than that of native catalase [22]. After binding, internalisation of antibodies takes place with trafficking of catalase into the pulmonary endothelium. Biotinylation of catalase has been shown to elevate and prolong serum levels but does not increase pulmonary uptake. Conjugation of catalase with mouse IgG did not increase pulmonary uptake of catalase [22].

These and supporting data from our other work clearly demonstrate that lung endothelial immunotargeting of catalase conjugated with anti-ACE antibodies is possible. This work provides a real opportunity for translation of laboratory studies on animals into clinical settings in the near future. Our group recently developed a method for perfusion of human lung resections to test anti-ACE mAbs and their accumulation in the human lung under in vivo conditions [25]. Results within this setting and in a large animal model, in addition, are needed before introduction in a clinical setting.

References