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sCR1sLe^X reduces lung allograft ischemia–reperfusion injury but does not ameliorate acute rejection ☆

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Abstract

Background: Combined inhibition of complement and leukocyte adhesion by sCR1sLe^X reduces lung allograft dysfunction up to 24 h. In the present study its effect on graft function and acute rejection was evaluated up to 5 days after experimental transplantation. **Methods**: Orthotopic single left lung transplantation was performed in 35 male rats (Brown Norway to Fischer 344) after a total ischemic time of 20 h. Two groups were assessed after 1, 3, and 5 days post-transplant, respectively ($n = 5$ per group and time point): controls vs. recipients which received 10 mg/kg sCR1sLe^X 15 min prior to reperfusion. In addition, five animals received 10 mg/kg per day sCR1sLe^X for 5 days. For blood gas analysis of the graft, the contralateral lung was occluded for 5 min to assess graft function. Lung grafts were flushed, and histological grading was performed in blinded fashion according to the International Society for Heart and Lung Transplantation criteria. **Results**: Graft PaO_2 in recipients treated with sCR1sLe^X was superior on day 1 (383 ± 118 vs. 56 ± 15 mmHg; $P < 0.0001$) and day 3 (446 ± 48 vs. 231 ± 108 mmHg; $P < 0.0001$). Five days after transplantation, no difference in PaO_2 was found (61 ± 28 vs. 83 ± 31 mmHg; $P = 0.59$). Repeated treatment with sCR1sLe^X for 5 days did not improve PaO_2 (64 ± 5 mmHg; $P = 0.65$ vs. control; $P = 0.93$ vs. sCR1sLe^X). At any time point, there was no difference in the degree of rejection between groups. **Conclusions**: In this model sCR1sLe^X provided marked improvement of graft function up to 3 days, but inhibition of both complement system and selectin dependent leukocyte adhesion failed to protect against acute rejection.

Keywords: sCR1sLe^X; Lung transplantation; Reperfusion injury; Graft rejection; Models; Animal; Complement

1. Introduction

Lung transplantation has become an established therapeutic option for end-stage pulmonary disease. Ischemia and reperfusion injury remains the major problem in the early phase after lung transplantation. Both severe reperfusion injury and acute rejection episodes may predispose to chronic graft rejection, i.e. obliterative bronchiolitis. Improved preservation and the flushing technique have reduced morbidity of early graft dysfunction, however, severe ischemia–reperfusion injury still occurs in about 10% of lung transplant recipients.

The pathophysiology of ischemia–reperfusion injury has been extensively studied. Therapeutic strategies of blocking only one of the redundant pathways of the nonspecific immune response have shown limited success [1]. Therefore, the modulation of more than one pathway of ischemia–reperfusion injury seems to be a promising strategy.

Recently, the glycoprotein sCR1sLe^X (Avant Immunotherapeutics, Needham, MA) has been synthesized by post-translational glycosylation of recombinant human soluble complement receptor type 1 (sCR1) with sialyl Lewis X (sLe^X; CD15s) in a mammal cell line [2]. sCR1sLe^X combines the effects of both sCR1 and sLe^X in one molecule. sCR1 is the most potent known inhibitor of the three complement pathways [3]. sLe^X is a terminal component of oligosaccharides on many glycoproteins and glycolipids on leukocytes and endothelial cells and a chief ligand common to all selectins. Its biological potential has been shown by a dramatic reduction of lung injury after intravenous infusion of cobra venom factor, an injury that is dependent on neutrophils, oxygen radicals, and P-selectin [4], and its effectiveness has been confirmed in further lung injury models [5]. sCR1sLe^X reduced myocardial infarct

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size [6] and neutrophil infiltration in acute lung injury models in vivo [7] as well as infarct size and consecutive neurologic deficit in experimental stroke in mice. Its effect has been demonstrated not only when sCR1sLe^X has been given as pretreatment, but also when the drug has been administered after the onset of ischemia [8]. In these models sCR1sLe^X was superior to sCR1 in several aspects.

The aim of this study was to evaluate the effect of sCR1sLe^X on post-transplant graft function in a rat model of left lung allotransplantation of major immunological mismatch after prolonged ischemia in comparison with untreated controls. The extent of immunological protection was specifically addressed by this study design, as in patients such combination of reperfusion injury and immunological mismatch is usually combined and therefore of high clinical relevance. Furthermore, continuous daily treatment was compared to single drug application before reperfusion.

2. Materials and methods

Weight matched (200–250 g) male Fischer F344 rats received orthotopic single left lung allografts from male Brown Norway rats. A cuff technique for the vessel anastomoses and a conventional running suture for the bronchial anastomosis were applied. All animals received humane care in compliance with the European Convention of Animal Care. The protocol was approved by the local animals study committee.

2.1. Donor procedure

Animals were anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg) and heparinized (500 IU/kg). A tracheotomy was carried out and the animals were ventilated through a cannula ($FiO_2 = 1.0$) by a Harvard rodent ventilator (Harvard Apparatus, South Natick, MA) at a tidal volume of 10 ml/kg. After division of the inferior vena cava and resection of the left appendix of the heart, a small silicon tube was inserted into the main pulmonary artery. Both lungs were flushed with 20 ml of LPD solution (Perfadex®, Xvivo, Göteborg, Sweden) at a pressure of 20 cm H_2O. The trachea was tied in end-inspiration. The heart–lung block was removed and 14 gauge cuffs were placed around the pulmonary artery and vein. The vessels were inverted and tied onto the cuff. The lung was stored in LPD solution at 4 °C until implantation.

2.2. Recipient procedure

Transplantation was performed after 20 h of cold ischemia at 4 °C. The recipient was anesthetized by breathing Halothane in a glass chamber followed by intubation. Anesthesia was maintained with Halothane 2%. A left lateral thoracotomy was performed in the 4th intercostal space. The left hilum was dissected. After clamping the pulmonary artery and vein with removable microvascular clips, the pulmonary vein was opened, flushed with heparinized saline solution, and the cuff was inserted and fixed with 6-0 Silk. In the same technique, the pulmonary artery was anastomosed. The native left lung was removed and the bronchial anastomosis performed with a running over-and-over suture with 9-0 Monosof® (Tyco Healthcare, Wollerau, Switzerland). The lung was first reventilated and then reperfused. A chest tube was inserted and the thoracotomy closed. The chest tube was removed after restoration of spontaneous breathing.

2.3. Assessment

The recipient animal was anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg) and ventilated with an FiO_2 of 1.0, a frequency of 100/min and a tidal volume of 8 ml/kg by a tracheotomy. For functional assessment of the transplanted left lung, the right hilum was dissected and the right pulmonary artery and the right main bronchus were occluded with microvascular clips. Five minutes after occlusion, a steady state was reached and an arterial blood gas sample was drawn from the thoracic aorta which was assessed with a blood gas analyzer (AVL 993, AVL List GmbH, Graz, Austria). After heparinization with 500 IU/kg, the microvascular clips were removed and the lungs were flushed with 20 ml saline solution through the pulmonary artery. The heart–lung block was excised and the lungs were fixed overnight at room temperature with 10% buffered formalin. Formalin was instilled through a tube inserted in the trachea to expand the lungs with a defined pressure of 20 cm H_2O. The transplanted left lung and the native right lung were then separately embedded in paraffin, and slides of 4 μm thickness were stained with hematoxylin–eosin. The slides were rated by a lung pathologist in blinded fashion according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT) [9].

2.4. Study groups

In each group five animals were transplanted for each time interval until harvest, i.e. days 1, 3 and 5, respectively. In treated animals, recipients received 10 mg/kg sCR1sLe^X 15 min prior to reperfusion by intracardiac injection. In addition, five recipients were treated continuously with daily intravenous injection of 10 mg/kg sCR1sLe^X for 5 days.

2.5. Statistical analysis

For PaO_2, the mean ± standard deviation and for histological grading, the median (range) is given. Analysis of variance (ANOVA) with planned contrast analysis between the groups (PaO_2) and the Mann–Whitney *U*-test (histological grading) were applied, respectively. The STATIS-

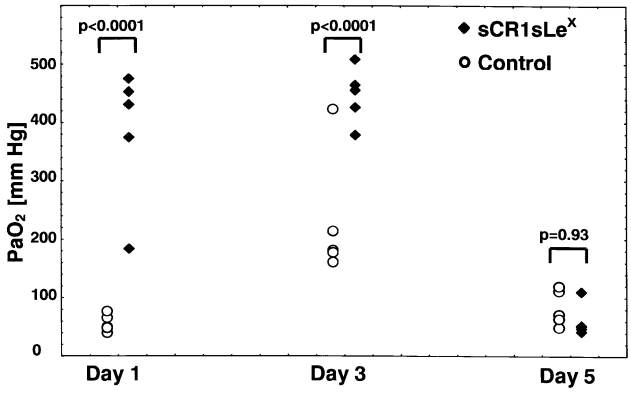

Fig. 1. PaO_2 (mmHg) of the isolated grafts at 1, 3, and 5 days after reperfusion (open circles: control animals; black squares: $sCR1sLe^X$).

TICA 5.1 software (StatSoft®, Tulsa, OK) was used. A P value of less than 0.05 was considered significant.

3. Results

Warm ischemic time in all transplantation groups was between 20.0 ± 0.71 and 20.8 ± 1.30 min, with no significant difference between groups.

Four recipients in the control group and one animal treated with $sCR1sLe^X$ suffered from severe edema with aspiration to the contralateral side and died within the first 3 h after transplantation. In addition, one recipient died due to technical problems regarding the bronchial anastomosis. All these animals were excluded from analysis, and further transplantations were carried out.

3.1. Blood gas analysis

Twenty-four hours after reperfusion, PaO_2 was very low in control animals (56 ± 15 mmHg). Treatment with $sCR1sLe^X$ resulted in superior graft function compared to controls (383 ± 118 mmHg; $P < 0.0001$; Fig. 1).

A further improvement in PaO_2 was noted on day 3 in treated animals (446 ± 48 mmHg) whereas arterial oxygen pressure in controls was 231 ± 109 mmHg ($P < 0.0001$ vs. $sCR1sLe^X$).

No significant difference between controls and treated animals was observed 5 days after transplantation (83 ± 31 vs. 61 ± 28 mmHg; $P = 0.59$).

Daily treatment with $sCR1sLe^X$ for 5 days did not reveal any improvement (64 ± 5 mmHg) compared to either controls on day 5 ($P = 0.65$) or animals which received a single dose of $sCR1sLe^X$ and were sacrificed after 5 days ($P = 0.93$).

Intra-group analysis in controls revealed an improvement of PaO_2 from day 1 (56 ± 15 mmHg) to day 3 (231 ± 109 mmHg; $P < 0.0001$). No significant difference was observed between day 1 and day 5 (83 ± 31 mmHg; $P = 0.52$). The superior graft function 24 h after reperfusion in recipients treated with $sCR1sLe^X$ (383 ± 118 mmHg) was followed by a small increase of PaO_2 on day 3 (446 ± 48 mmHg; $P = 0.14$). In this allograft setting, PaO_2 on day 5 in $sCR1sLe^X$ treated animals (61 ± 28 mmHg) was very low compared to day 1 ($P < 0.0001$).

3.2. Rejection grading

No difference in either perivascular or peribronchial rejection grading between controls and treated animals was observed at any given point in time (Table 1). In both groups, the onset of rejection was seen already on day 3 (control: $P = 0.056$ (perivascular), $P = 0.11$ (peribronchial) vs. control day 1; $sCR1sLe^X$ group: $P = 0.0079$ (perivascular), $P = 0.15$ (peribronchial) vs. $sCR1sLe^X$ group day 1), and significant rejection was noted 5 days after transplantation (control: $P = 0.016$ (perivascular), $P = 0.029$ (peribronchial) vs. control day 1; $sCR1sLe^X$ group: $P = 0.0079$ (perivascular), $P = 0.0079$ (peribronchial) vs. $sCR1sLe^X$ group day 1).

4. Discussion

In this model of unilateral left lung allotransplantation in rats after prolonged ischemia, inhibition of both the complement system and the selectin dependent leukocyte adhesion by $sCR1sLe^X$ exerted prolonged protection against reperfusion injury as substantiated by the transplants' gas exchange at days 1 and 3 after transplantation. However, no effect on acute rejection has been observed.

A recent study by our group underlined the significant improvement by $sCR1sLe^X$ after prolonged ischemia on graft function at 24 h after transplantation of both gas exchange and markers of reperfusion injury compared to either untreated controls or recipients treated with the complement inhibitor sCR1 alone [10].

Complement, a proteolytic cascade system, is an effector of the non-specific and humoral immune response and a stimulator of leukocyte activation by the complement

Table 1
Rejection grading of the allografts 1, 3, and 5 days after reperfusion

	Day 1		Day 3		Day 5	
	Vascular	Airway	Vascular	Airway	Vascular	Airway
Control	A0 (A0–A2)	B0 (B0–B1)	A2 (A1–A2)	B1 (B0–B2)	A3 (A2–A4)	B2 (B2–B2)
$sCR1sLe^X$	A0 (A0–A2)	B0 (B0–B0)	A2 (A2–A2)	B1 (B0–B2)	A3 (A3–A3)	B2 (B2–B3)

component 5a (C5a). The complement receptor type 1 (CR1; CD35; C3b/C4b receptor) is a transmembrane glycoprotein on erythrocytes and virtually all leukocytes. Whereas phagocytes bind particles by CR1 when they are coated with activated complement component 3 (C3b) and subsequently ingest them, the extracellular portion of CR1 (soluble CR1; sCR1) can be shed from neutrophils or macrophages [3,11]. sCR1 has been shown to be the most potent inhibitor of the classical, alternative, and lectin pathway of complement activation with more than 100 fold more effect than any other soluble complement regulatory protein [3].

Ischemia–reperfusion injury has been shown to be complement dependent, because inhibitors of complement activation limited this type of injury, e.g. in models of rat myocardial infarction [12], acute neutrophil dependent inflammatory lung injury [13] or iso- and allograft transplantation [14,15]. In rat lung isotransplantation, sCR1 conferred protection against lung injury. It reduced neutrophil infiltration, cellular deposition of C5b-9 complexes and serum complement hemolytic activity, and improved pulmonary vascular resistance, gas exchange and ultimately survival [15]. In unilateral swine lung allotransplantation after prolonged ischemia, sCR1 completely inhibited serum complement activity and significantly reduced reperfusion edema [16]. The relevance of these findings has recently been confirmed by a clinical multicenter trial with sCR1 in lung transplant patients [17].

Moreover, complement inhibition may also reduce the acquired immune response, as the induction of antibody responses against T cell dependent antigens is modulated by complement. Alloantibody response against donor-specific antigens and the proportion of activated B and T splenocytes after transplantation were decreased by complement inhibition [18], endothelial and vascular injury was reduced, and graft survival in experimental kidney allotransplantation was prolonged [14].

Selectins are three closely related and well conserved adhesion molecules that mediate initial leukocyte endothelial interaction. They interact with fucosylated carbohydrate ligands, especially structures containing sLe^X, and are upregulated in a number of different lung injuries. Specific inhibitors such as monoclonal antibodies, selectin ligands [19] or inducers of selectin shedding such as leumedins have been studied in different models of lung transplantation. Blockade of both leukocyte integrin adhesion molecule and its counterpart, intercellular adhesion molecule-1 (ICAM-1), has been shown to be efficient in a rat lung transplant model, as combined administration of monoclonal antibodies against ICAM-1, CD11a and CD18 resulted in superior gas exchange 24 h after reperfusion and reduced neutrophil accumulation in lung tissue [20]. In addition, blockade of P-selectin by a monoclonal anti-P-selectin antibody or a selectin inhibitor improved graft function and reduced PMN infiltration after syngeneic rat lung transplantation [21].

In vivo, selectin ligands are usually necessary to recruit neutrophils to sites of inflammation, evidenced by the congenital disorder of leukocyte adhesion deficiency syndrome type 2 where patients are deficient of sLe^X expression [22]. sLe^X is a terminal component of oligosaccharides on many glycoproteins and glycolipids on leukocytes and endothelial cells, a chief ligand common to all selectins and therefore proved to be an attractive 'mimic' to inhibit selectin dependent injury [4]. Endothelial sLe^X may be upregulated in the graft endothelium within 30 min post-revascularization as recently shown in kidney grafts [23], and administration of the sLe^X analogue CY-1503 improved gas exchange after canine lung allotransplantation and reduced neutrophil influx to the graft tissue and alveoli [24].

The glycoprotein $sCR1sLe^X$ has been synthesized by glycosylating sCR1 with the tetrasaccharid sLe^X [2], thus maintaining the complement blocking activity of sCR1 and furthermore blocking selectin-mediated cellular adhesion. The main counterparts of endothelial selectins, E-and P-selectin, are blocked, as well as the ligands of platelets P-selectin and leukocytes L-selectin [7]. A further advantage may be that $sCR1sLe^X$ accumulates in inflamed areas through binding to endothelial selectins [7]. In vivo models of experimental stroke [8], myocardial infarction [6] and neutrophil dependent acute lung injury [7] demonstrated that $sCR1sLe^X$ efficiently inhibited complement. In the stroke model the administration of $sCR1sLe^X$ at the time of reperfusion also improved outcome, albeit to a lesser degree. Treating evolving reperfusion injury may therefore be a promising option for clinical use of this substance.

Acute vascular rejection was uniformly histologically observed in our model and confirmed by the severely compromised gas exchange of the grafts in both treatment groups as well as in the controls. Neither single shot treatment on day 1, nor daily continuous treatment until day 5 reduced acute rejection in this model. This finding is in contrast with protective effects in allotransplantation by either blockade of complement [25] by sCR1 or of selectin by sLe^X in a similar rat lung transplant model [26]. The substance did not reduce the primarily lymphocytic inflammation of acute rejection although it reduced neutrophil dependent ischemia–reperfusion in the utilized model. The results suggest insufficient blocking of acute lung rejection, e.g. insufficient dosage, or substance inactivation. There seems to be evidence that $sCR1sLe^X$ binds to inflamed vascular endothelium, and dosage may be inadequate for the large inflamed surface during lung rejection. However, the possibility that T-lymphocyte traffic during rejection is less dependent on selectins cannot be completely excluded. A concomitant immunosuppressive regimen might have led to differences between treatment groups in this model of major histocompatibility mismatch and needs further evaluation.

In conclusion, $sCR1sLe^X$ has proven to have a remarkable efficacy after prolonged ischemia to inhibit ischemia–

reperfusion lung injury up to 3 days after rat lung allotransplantation. Further studies should focus on its effect in already established reperfusion injury. As a sole anti-rejection substance, sCR1sLe^X, however, failed to protect against severe acute rejection.

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