

**Keratinocyte growth factor prevents intraalveolar oedema in
experimental lung isografts**

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Short title: KGF protects rat lung isografts

Abstract:

Primary graft dysfunction, characterized by intraalveolar oedema, is a major obstacle in pulmonary transplantation. We evaluate the potential of keratinocyte growth factor (palmiferin, Δ N23-KGF) to prevent oedema in lung transplants.

Intratracheal instillation of 5 mg/kg Δ N23-KGF was performed in Lewis rats on days 3 and 2 before explantation. Control animals obtained an equivalent volume of vehicle. Left lungs were isogeneically transplanted and the graft recipients were sacrificed one day later for stereological analysis of intraalveolar oedema and bronchoalveolar lavage. The total protein and phospholipid content as well as surfactant proteins were measured. Surfactant activity was analyzed with a pulsating bubble surfactometer.

In grafts from control treated donors, the fraction of intraalveolar oedema amounted to $3.4 \pm 1.1\%$ of the total parenchymal volume. Treatment of donor lungs with Δ N23-KGF reduced oedema to a fraction of $1.6 \pm 0.8\%$. In the lavage fluid of pulmonary grafts from Δ N23-KGF-treated donors, the total protein content was decreased compared to vehicle-treated lung transplants, whereas phospholipids did not differ. The protein fraction contained increased amounts of surfactant protein-C after Δ N23-KGF-treatment and surfactant function was improved.

Treatment of donor lungs with palmiferin protects against intraalveolar oedema formation upon transplantation. This effect appears to be mediated by an improved surfactant homeostasis.

Key words: Keratinocyte growth factor, transplantation, oedema, lung, primary graft dysfunction

Introduction

Transplantation is the only curative therapy for numerous patients suffering from end-stage lung diseases. During the first 3 months posttransplantation, about 13% of pulmonary allograft recipients die [1]. Primary lung graft dysfunction (PGD) and infections are the main reasons for this poor survival early after transplantation and both conditions frequently coexist [1, 2]. According to a recently proposed consensus definition, PGD is characterized by hypoxemia and radiographic evidence of diffuse alveolar infiltrates [3]. Histopathological correlates of this syndrome are non-specific alveolar damage and lung oedema [4].

Donor treatment with keratinocyte growth factor (KGF, fibroblast growth factor 7) might help to prevent PGD in pulmonary graft recipients because KGF protects the lung in various models of acute lung injury including hyperoxia, bleomycin, acid aspiration, mechanical injury, radiation, infections, and even graft-versus-host disease [for review: 5]. Comparable to PGD, these models of lung damage involve pulmonary oedema formation. Lung protection by KGF is mediated by several mechanisms: Most importantly, KGF stimulates the proliferation of alveolar epithelial type II cells (AEIIs) and results in a pronounced AEII hyperplasia on days 2 and 3 after treatment [6, 7]. The barrier function of both the alveolar epithelium and the endothelium [8] as well as the capacity of fluid reabsorption from the alveolar space is improved [9]. KGF increases surfactant protein and phospholipid levels in vitro and in vivo [10-12]. Donor treatment with exogenous surfactant has been shown to be effective in preventing PGD in experimental settings [13-15] as well as in a small clinical trial [16]. Stimulation of the endogenous surfactant system by the application of KGF appears as an appealing approach to prevent lung transplantation associated reperfusion injury.

The use of KGF or the more stable N-terminally truncated analogue Δ N23-KGF (palifermin) [17] in the context of transplantation of lungs from deceased donors is limited by the fact that the lungs are most effectively protected when treated with KGF 2 to 3 days before the injurious stimulus is applied. Lung protection by KGF might, however, be useful in living donor

lobar lung transplantation which is performed in pediatric patients to mitigate the growing competition for deceased donor lungs [18]. As lung regeneration is also stimulated by KGF [19], both graft donor and recipient might profit from KGF-application to donor lungs.

The aim of this study was to evaluate the potential of donor lung treatment with Δ N23-KGF to prevent alveolar oedema formation after transplantation. We use an experimental rat model of isogeneic orthotopic left lung transplantation involving short ischemic times comparable to the situation in living donor lung transplantation.

Materials and Methods

Study design

Lung protection by KGF is only effective when the growth factor is applied 2 to 3 days before injury [5]. In contrast to living donor lungs, grafts from deceased donors can probably not be protected against PGD. Therefore, we chose an experimental design resembling the living donor procedure where ischemic times are kept as short as possible.

Inbred Lewis (LEW) donor rats were treated on days 1 and 2 with bioactive recombinant human Δ N23-KGF or vehicle via intratracheal instillation. On day 4, orthotopic left lung transplantation to isogeneic recipients was performed. Warm and cold ischemic times were kept as short as possible. Oedema formation was evaluated by stereological methods, by protein and surfactant analysis in the bronchoalveolar lavage (BAL) fluid 24 h after transplantation. This point in time was chosen, because in this experimental model, intraalveolar oedema was obvious one day after transplantation which resolved on days 2 and 3 [20].

Animals

Male LEW (RT1^l) rats were raised under pathogen free conditions according to the FELASA standards and provided by Harlan Winkelmann (Borchen, Germany). Animal care and animal experiments were performed following

the current version of the German Law on the Protection of Animals as well as the NIH principles of laboratory animal care.

KGF-treatment and lung transplantation

Bioactive, purified, endotoxin free Δ N23-KGF (palifermin) produced in *Escherichia coli* was provided by Amgen (Thousand Oaks, California, USA). On day 1, animals weighing 200-270 g were anaesthetized by short time inhalation of isofluran (Forene, Abbot, Wiesbaden, Germany), intubated orally with a 16 G intravascular catheter (Insyte, Beckton Dickinson, Sandy, USA), and instilled via the trachea with 5 mg Δ N23-KGF per kg body weight or an equivalent volume (200-270 μ l) of phosphate buffered saline (PBS). The treatment was repeated on day 2. On day 4, these rats served as donors for lung transplantation.

Orthotopic left lung transplantation was performed in the isogeneic LEW to LEW rat strain combination as described [20]. In brief, a cuff technique was used for the vascular anastomoses and sutures with interrupted stitches for the bronchial anastomoses. Warm ischemic times remained below 20 min and cold ischemic times below 25 min. No immunosuppression was given.

Pulmonary grafts were harvested 24 h after transplantation for histology or BAL. Only grafts which were ventilated and supplied with blood were considered to be technically successful and included in this study.

Graft histology

The lungs were flushed in situ with 20 ml saline via the pulmonary artery and fixed by vascular perfusion with 1.5% glutaraldehyde, 1.5% paraformaldehyde dissolved in 0.15 M HEPES buffer, pH 7.4, at a hydrostatic pressure of 15 cm H₂O for 20 min while the airway pressure was adjusted to 12 cm H₂O. Thereafter, the trachea was ligated and the heart-lung block was removed from the recipient rat. Fixation by perfusion was followed by 24 h immersion in the same fixative at 4° C.

After determination of the lung volume by fluid displacement, lungs were embedded into 2% aqueous agar-agar at 40°C, cooled to room temperature,

stored at +8°C for 2-3 hours, and then cut into 4 mm thick slices by means of a tissue slicer with the first cut being at a random position within the first 4 mm. The slices were post-fixed with 1% osmium tetroxide in 0.1 M Na-cacodylate buffer followed by overnight incubation in half-saturated aqueous uranyl acetate. Slices were dehydrated through a series of graded acetone, and embedded into glycol methacrylate (Technovit 7100, Kulzer, Wehrheim, Germany). After staining of semi-thin sections with Methylene blue/Azur II, intraalveolar oedema was determined by stereological analysis.

Stereological analysis

Intraalveolar oedema was determined by point counting according to a two-level cascade sampling procedure [21] using a light microscope (Olympus BX51, Olympus, Kobenhavn, Denmark) equipped with a computer-assisted stereology toolbox (CAST Grid, Visiopharm, Hershholm, Denmark). At level 1, the volume densities of non-parenchyma (airways, arteries, veins, pleura) and parenchyma (alveolar septa, airspace of alveoli and alveolar ducts) were estimated at a primary magnification of 4 x. At level 2, the volume densities of alveolar and alveolar ducts airspace (occupied by air or oedema fluid), alveolar septal tissue, and capillary lumen were estimated at a primary magnification of 20 x. At each level, a set of systematic uniform random sampled fields of view were collected for point counting. Volume densities, fractions of total lung volume, and absolute volumes of each compartment were calculated as described previously [22]. Absolute volumes of the transplants and the right native lungs were normalized to body weight of the donors and the recipient rats respectively and are given as mass-specific volumes.

Bronchoalveolar lavage

Pulmonary graft recipients were anaesthetized and the trachea was cannulated. After sternotomy, the right main bronchi were clamped and the left transplanted lungs were lavaged 5 times with 2 ml saline, 37° C, each. Thereafter, the right lungs were lavaged 5 times with 3 ml saline each. BAL

fluid (BALF) was collected on ice, centrifuged for 15 min at 300 g and stored at -20° C until further analysis.

Analysis of bronchoalveolar lavage fluid

Total proteins were quantified in BALF using a commercial assay kit (BCA, Pierce, Bonn, Germany). The content of the hydrophobic surfactant proteins SP-B and SP-C in the BALF was determined by ELISA as described [23, 24].

BALF was centrifuged at 48,000 x g (1 hour, 4 °C) using a Beckman L5-50 centrifuge (type 40 rotor) to isolate large surfactant aggregates (LA). The LA-containing pellets were resuspended in 0.9 % NaCl and the relative LA content was assessed by relating the fraction of pelleted PL to the total amount of PL. Lipids were extracted from original BALF or the isolated LA fraction with chloroform/methanol according to the method of Bligh and Dyer [25] and organic phases were taken for quantification. Phospholipids were quantified by means of a colorimetric phosphorus assay as described [26].

Measurement of surfactant surface activity

Surface activity of surfactant material was measured with a pulsating bubble surfactometer [27]. Experiments were performed at hypophase concentrations of 1 mg phospholipids per ml. Briefly, 40 µl of the resuspended LA pellet were injected into the bubble chamber. Before starting bubble pulsation the material was allowed to stabilize for 5 min. Measurements were performed with a cycle speed of 20 cycles/min for 5 min. Prior to bubble oscillation, phospholipid adsorption (γ_{ads}) was measured as the surface tension of the originated bubble after a time period of 10 sec. Minimum surface tension at minimum radius of the bubble after 5 min of pulsation was defined as γ_{min} . Respectively, maximum surface tension at maximum radius was defined as γ_{max} . Data were digitalized and recorded by computer.

Statistical analysis

Data are given as mean \pm standard deviation (SD) in the text, are presented in box plots in graphs, and were analyzed with non-parametric Kruskal-Wallis test followed by Mann-Whitney rank sum test using SPSS software (Munich, Germany) with $p \leq 0.05$ set as level for significance.

Results

Transplantation of Δ N23-KGF-treated lungs

LEW donor rats were treated on days 1 and 2 with Δ N23-KGF via intratracheal instillation, control donors obtained an equivalent volume of PBS. Lungs were transplanted on day 4 and harvested 24 h thereafter. The technical success rate was identical in both experimental groups: 86% for PBS-treated donors (n=28) and 85% for Δ N23-KGF-treated donors (n=40).

Graft histopathology

In transplants from PBS-treated donors, intraalveolar oedema could be detected (Fig. 1a). Alveoli containing protein-rich oedema fluid formed clusters surrounded by unimpaired lung parenchyma. Frequently, a quarter to half of the alveolar area was filled with oedema which lined the alveolar wall (Fig. 1a). However, no severe damage of the lung parenchyma was seen. As described before for lung isografts on day 1 posttransplantation, the connective tissue surrounding blood vessels exhibited oedematous swelling and contained some mononuclear leukocytes [20].

Lung isografts originating from donors which were treated with Δ N23-KGF (palifermin), revealed pronounced hyperplasia of AEIIs (Fig 1b). This is a typical feature of rat lungs treated with KGF indicating that the treatment was successful [5]. In comparison to control pulmonary transplants, the area of the intraalveolar space filled with oedema was reduced (Fig. 1b). The perivascular oedema and leukocytic infiltrate in KGF-treated pulmonary isografts did not differ from PBS-treated isografts.

Stereological analysis of intraalveolar oedema

To quantify the intraalveolar oedema in vehicle-treated and KGF-treated lungs, stereological analysis was performed (Table 1 and Fig. 1c).

In isografts originating from PBS-treated donors, the fraction of intraalveolar oedema amounted to $3.4 \pm 1.1\%$ of total parenchymal volume. Pretreatment of donor lungs with Δ N23-KGF significantly ($p=0.005$) reduced the oedema to a fraction of $1.6 \pm 0.8\%$ (Fig. 1c). Relating intraalveolar oedema volume to the alveolar air-blood barrier, oedema amounts to $41.1 \pm 17.8\%$ of tissue volume in transplanted lungs of PBS-treated rats versus $16.6\% \pm 9.8\%$ after of Δ N23-KGF-treatment ($p=0.005$). Mass-specific absolute oedema volume was also significantly reduced (Table 1). The native right lung was included in this analysis as an internal control (Table 2). No differences were observed between the right native lungs from recipients of PBS-treated or Δ N23-KGF-treated left lungs.

Analysis of bronchoalveolar lavage fluid

PBS-treated or Δ N23-KGF-treated grafts and the native right lung were lavaged separately. In addition, lungs from sex and age matched healthy LEW rats were included.

Transplantation of PBS-treated lungs resulted in a significant ($p \leq 0.01$) increase in the total protein content in the BALF from 2.9 ± 0.2 mg per left lung in healthy controls to 12.9 ± 4.2 mg per graft (Fig. 2). Application of Δ N23-KGF before transplantation reduced the amount of protein to 6.4 ± 1.4 mg per graft ($p \leq 0.01$). The protein content of the right native lungs of all experimental groups remained at about the same level.

SP-B and SP-C were measured in the BALF by ELISA. The total SP-B content in BALF from left control lungs, PBS-treated grafts and Δ N23-KGF-treated grafts was identical. The same was true for the right native lungs. Due to the increase in the total protein and phospholipid content upon lung transplantation, the SP-B/total protein ratio decreased but no significant differences were obvious between PBS-treated and Δ N23-KGF-treated pulmonary isografts (Fig. 2).

In contrast, the total SP-C content in BALF was higher in grafts compared to healthy control left lungs ($p \leq 0.05$). No significant difference was seen among PBS-treated and $\Delta N23$ -KGF-treated grafts, or among the right lungs from all experimental groups, respectively. As expected, the SP-C/total protein ratio was decreased ($p \leq 0.05$) in PBS-treated transplants. In BALF obtained from $\Delta N23$ -KGF-treated grafts, however, the SP-C/total protein ratio did not differ from native control lungs and was higher ($p \leq 0.01$) when compared to the ratios measured in PBS-treated grafts (Fig. 2).

Transplantation increased the amount of phospholipids harvested from the left lung by BAL ($p \leq 0.01$). Among the right native lungs no changes were seen. $\Delta N23$ -KGF-treatment did not influence the phospholipid content in comparison to PBS-treated grafts. Consequently, the phospholipid/protein ratio decreased ($p \leq 0.01$) in PBS-treated pulmonary transplants, whereas in isografts from $\Delta N23$ -KGF-treated donors, this ratio did not differ from healthy control lungs (Fig. 2). The difference between PBS-treated grafts and $\Delta N23$ -KGF-treated grafts was statistically significant ($p \leq 0.05$). LA content was slightly increased in $\Delta N23$ -KGF-treated lungs, but did not statistically differ from PBS-treated lungs ($93.2 \pm 11,0$ vs $82,2 \pm 15,6$).

Surfactant activity

Surfactant activity was measured with a pulsating bubble surfactometer. The results are summarized in Table 3. The surface tension (γ_{ads} , γ_{min} and γ_{max}) of the isolated surfactant tended to be reduced in $\Delta N23$ -KGF-treated transplants in comparison to PBS-treated grafts. This difference was statistically significant ($p \leq 0.05$) for γ_{max} . A similar effect was also seen for the right native lung of recipients of $\Delta N23$ -KGF-treated lungs in comparison to PBS-treated lungs.

Discussion

We demonstrate that lungs from $\Delta N23$ -KGF-treated donor rats can be successfully transplanted. In addition, treatment of donor lungs with $\Delta N23$ -

KGF protects against perioperative lung injury which inevitably occurs during transplantation.

Impaired function of the air-blood barrier resulting in the formation of intraalveolar oedema is a hallmark of PGD [2]. Protection against PGD in lung grafts from Δ N23-KGF-treated donors compared to PBS-treated grafts was demonstrated in our study by independent techniques. First, we chose a stereological approach for quantification of lung oedema in lungs fixed by vascular perfusion, because this technique is more sensitive when compared to the analysis of the wet-to-dry weight ratio [22]. Sensitivity is important, because in our experimental setting involving short ischemic times, the amount of intraalveolar oedema is low. Furthermore, stereological data on lung oedema but not wet-to-dry weight ratio data have been shown to significantly correlate with lung function [22].

In a second approach, we measured the protein content in the BALF. According to published data [13], BALF from PBS-treated lung transplants contained more protein compared to healthy control lungs. Donor Δ N23-KGF-treatment, however, resulted in a significantly decreased protein content in the BALF of lung transplants.

A direct functional analysis of the lung transplants was not performed because the volume density of the intraalveolar oedema in PBS-treated grafts was too small to expect a severe impairment of blood oxygenation in comparison to Δ N23-KGF-treated lungs. A previous study investigating the effects of various preservation solutions on ischemia/reperfusion injury demonstrated that the oxygen tension measured during reperfusion was largely normal in rat lungs exhibiting a volume density of intraalveolar oedema of up 3%, but dropped sharply beyond 3% [22]. Although functional impairment can not be inferred as being severe in PBS-treated grafts (3.4% oedema), it can clearly be expected to be worse than in KGF-treated grafts (1.6% oedema). Such differences, however, might be obscured by the minor functional impairments that were observed after KGF-treatment [28].

A large body of literature describes improved surfactant homeostasis after lung treatment with KGF and implicates that, at least in part, protective

effects of KGF are mediated via surfactant [5, 29]. Surfactant function is impaired during the reimplantation response in rat lung transplants and instillation of surfactant improves early graft function [13]. Beneficial effects of exogenous surfactant application were additionally shown in mini-pigs [15], dogs [14], and in a small clinical study in human transplant patients [16]. We therefore analyzed the total phospholipid content of the BALF as well as SP-B and SP-C, small hydrophobic proteins that are most important in terms of biophysical functions of surfactant [30].

In line with previous studies [13], our results demonstrate that some characteristic parameters of pulmonary surfactant were changed upon lung transplantation in our experimental model: The total phospholipid content and the content in SP-C were increased in response to transplantation, whereas SP-B remained unchanged. However, the differences in the total amounts of these components were not significant between grafts from PBS- and Δ N23-KGF-treated donors, which may relate to the fact that the degree of injury was low in our experiments. The only parameter related to surfactant proteins which differed significantly was the SP-C/total protein ratio which might be functionally relevant because SP-C increases the resistance of surfactant to inhibition by serum proteins or oedema fluid [30].

Surfactant from Δ N23-KGF-treated pulmonary grafts tended to be more active compared to PBS-treated grafts with γ_{\max} being significantly different. In contrast to the protein content of the BAL fluid, surfactant function was also reduced in the right native lungs of graft recipients which might result from mechanical ventilation during surgery. In addition, the pulmonary graft appeared to contribute to the impairment of reduction function in the right native lung, since protection of the graft with Δ N23-KGF resulted in a better surfactant function in the native lung. This might be explained by a functional overload of the native lung in the presence of a damaged graft.

The time schedule to achieve effective lung protection by KGF-treatment was carefully optimized before by other authors [5]. Donor KGF-treatment 2 to 3 days before transplantation seems to be mandatory when aiming at a prevention of PGD. Therefore, KGF-treatment appears to be limited to

living donor lobar lung transplantation. However, pulmonary allografts are additionally damaged by postoperative mechanical ventilation, infections and rejection. Further experimental studies will be needed to evaluate if KGF also protects against these injuries. If so, recipients of lungs from deceased donors might also benefit from KGF-application in the perioperative or postoperative phase.

We conclude that donor lung pretreatment with palifermin reduced intraalveolar oedema, the histopathological correlate of PGD, in the rat lung transplantation model. In living donor lung transplantation, palifermin-treatment might be beneficial for donor and recipient as KGF has been evidenced to promote lung regeneration upon partial pneumonectomy [19]. We suggest that donor lung treatment with palifermin might be a promising strategy to prevent early graft dysfunction in humans, particularly when living donor transplantations are performed.

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Figure legends

Fig. 1: Histopathology of pulmonary isografts one day after transplantation. a) Semi-thin section of the lung parenchyma of a PBS-treated transplant stained with Methylene blue/Azur II. The arrows are pointing to intraalveolar oedema. b) Section of a pulmonary transplant which was pre-treated with keratinocyte growth factor (KGF; Δ N23-KGF). The alveolar walls are thickened due to alveolar epithelial type II cell hyperplasia. Intraalveolar oedema is rare. c) Volume fraction (% total parenchyma) of the intraalveolar oedema in lung transplants (tx) and right native lungs (nat) after PBS-treatment (PBS) or treatment with Δ N23-KGF. The box plots indicate median and percentiles 0, 25, 75 and 100. Small asterisks represent data beyond ± 3 x standard deviation; ** $p \leq 0.01$.

Fig. 1

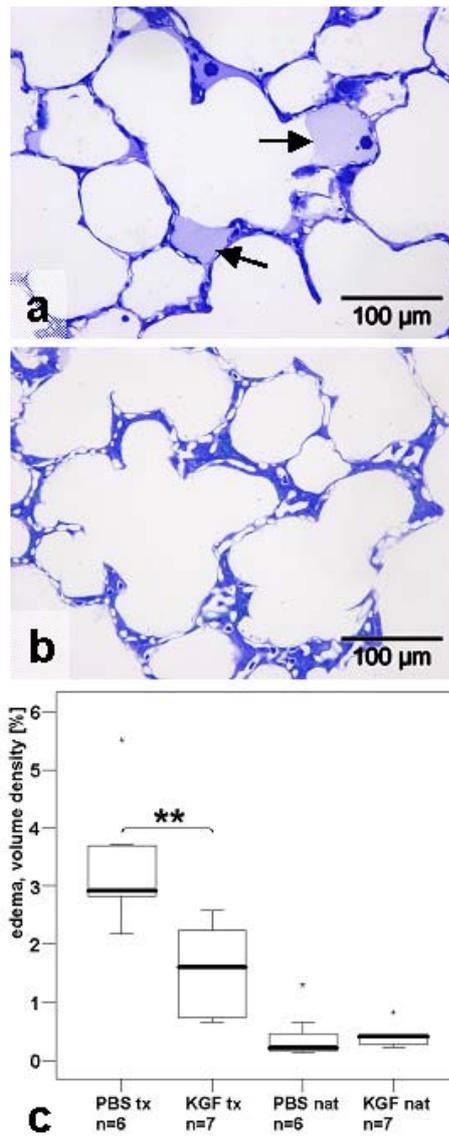


Fig. 2: Analysis of bronchoalveolar lavage (BAL) fluid of the left lungs and the right lungs of healthy controls and recipients of pulmonary isografts. Left lungs were transplanted and the right native lung of the recipients

remained in the recipient. The total protein and phospholipid content in the BAL was determined as well as the ratio of surfactant protein-B (SP-B) or SP-C and the total protein content. The box plots indicate median and percentiles 0, 25, 75 and 100. Small asterisks represent data beyond ± 3 x standard deviation; * $p \leq 0.05$, ** $p \leq 0.01$.

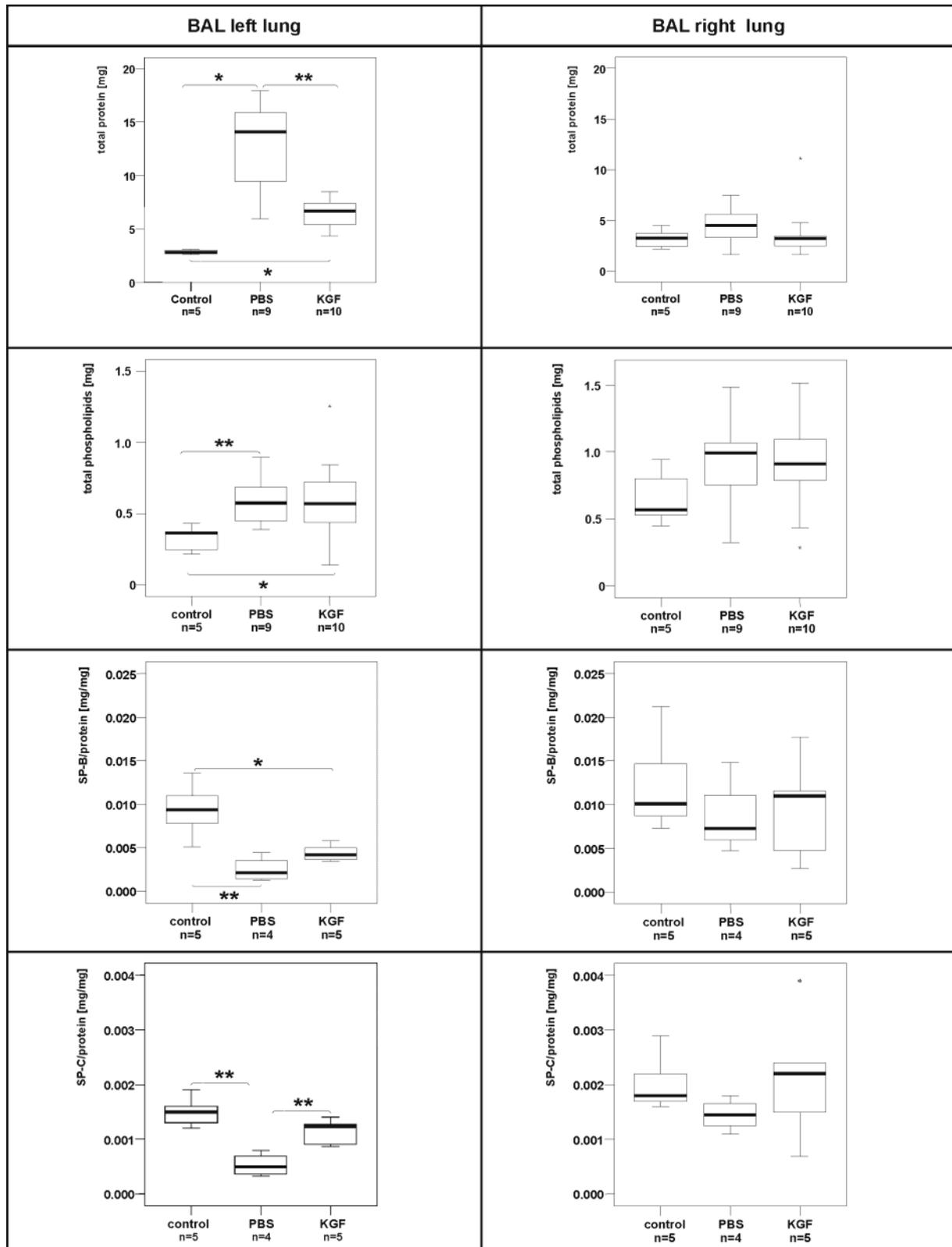


Table 1 – Results of Stereologic Analysis of Transplanted Left Lungs

Parameters	PBS-treated (n=7)	ΔN23-KGF-treated (n=6)
Body weight [g]	239.0 (2.2)	259.0 (14.0)*
Mass-specific volumes [mm³/g b.w.]		
Lung	11.1 (3.0)	12.7 (1.1)
Non-Parenchyma	1.3 (0.5)	1.4 (0.5)
Parenchyma	9.8 (2.5)	11.3 (1.1)
Air Space	8.2 (2.2)	9.6 (1.0)
Alveolar Oedema	0.32 (0.11)	0.18 (0.10)*
Septal Tissue	0.86 (0.35)	1.12 (0.17)
Capillary Lumen	0.38 (0.13)	0.42 (0.15)

Data are given as mean (SD); * indicates $p \leq 0.05$

Table 2 – Results of Stereologic Analysis of Native Right Recipient Lungs

Parameters	Recipient of PBS-treated lung (n=7)	Recipient of Δ N23-KGF-treated lung (n=6)
Body weight [g]	241.1 (1.4)	258.3 (5.5)*
Mass-specific volumes [mm³/g b.w.]		
Lung	18.7 (4.7)	22.3 (4.2)
Non-Parenchyma	2.3 (0.4)	2.3 (0.6)
Parenchyma	16.3 (4.5)	20.1 (3.9)
Air Space	14.6 (4.4)	17.5 (3.6)
Alveolar Oedema	0.06 (0.06)	0.08 (0.05)
Septal Tissue	1.2 (0.2)	1.6 (0.4)
Capillary Lumen	0.6 (0.1)	0.8 (0.4)

Data are given as mean (SD); * indicates $p \leq 0.05$

Table 3 – Surface activity of surfactant isolated from pulmonary transplants (tx) and native lungs (nat).

	γ_{ads} [mN/m]	γ_{min} [mN/m]	γ_{max} [mN/m]
PBS tx (n=4)	36.5 (10.7)	23.6 (8.8)	45.2 (7.0)
KGF tx (n=4)	28.2 (5.2)	18.1 (1.2)	35.2 (3.4)*
PBS nat (n=4)	30.8 (3.9)	16.9 (2.2)	40.1 (3.1)
KGF nat (n=4)	28.5 (9.0)	11.5 (5.7)	32.3 (4.2)#

Graft donors were either vehicle-treated (PBS) or treated with Δ N23-KGF (KGF). Data are given as mean (SD); * statistically significant difference between Δ N23-KGF-treated and PBS-treated lungs $p \leq 0.05$; # statistically significant difference between the right native lungs of recipients of Δ N23-KGF-treated and PBS-treated lungs $p \leq 0.05$