Liver Growth Factor Improves Pulmonary Fibrosis Secondary to Cadmium Administration in Rats

Lourdes Martínez-Galán, Laura del Puerto-Nevado, Sandra Pérez-Rial, Juan José Díaz-Gil, Nicolás González-Mangado, and Germán Peces-Barba

Liver Growth Factor (LGF) is a liver mitogen with regenerating and anti-fibrotic activity even at extrahepatic sites. We used LGF in a lung fibrosis model induced by cadmium chloride (CdCl₂), to study its antifibrotic capacity.

Methods: Forty-two male Wistar rats were administered a single dose of 0.5ml/rat of CdCl₂ 0.025% (n = 21) or the same volume of saline (control group, n = 21). After 35 days, once a lesion was established, we started a 3 week treatment with LGF, after which we determined lung function —inspiratory capacity (IC), lung compliance (CL), forced vital capacity (FVC) and expiratory flow at 75% (FEF75%)—, lung morphometry —alveolar internal area (AIA), mean linear intersection (LM)—, and collagen (both by Sirius red and hydroxyproline residues) and elastin contents.

Results: Pulmonary fibrosis in CdCl₂ rats was characterized by a marked decrease in pulmonary function with respect to healthy controls —reductions of 28% in IC, 38% in CL, 31% in FVC, and 54% in FEF75%— which was partially recovered after LGF injection —18% IC, 27% CL, 19% FVC and 35% FEF75%—; increase in collagen and elastin contents —165% and 76%, respectively, in CdCl₂ rats, versus 110% and 34% after LGF injection—; and increases in AIA and LM, partially inverted by LGF.

Conclusions: Together, these data seem to demonstrate that LGF is able to improve lung function and partially invert the increase in lung matrix proteins produced by CdCl₂ instillation.

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Introduction

Pulmonary fibrosis is a conditioned characterised by a progressive deterioration of the pulmonary function. There are several animal models which attempt to reproduce this condition; perhaps the most extended is bleomycin induction (a fibrogenic agent widely employed for the study of the initial stages of the condition) since it is inverted if the agent is not continuously administered. For the study of the fibrotic stages, the assessment should take place in the second and third weeks from the moment of induction. For this, there are more effective experimental models, such as cadmium chloride (CdCl₂) by single dose endotracheal induction in animals which causes pulmonary inflammation with fibrotic activity accompanied by an alveolar space enlargement recalling human centricinar emphysema, but with a predominance of fibrosis. This agent induces a functional pattern characterised by a decrease in volume, pulmonary distension and expiratory flow as well as an increase in proteins on the lung matrix, collagen and elastin. Subsequent studies have also proven that the LGF is able to reduce both fibrosis in the carotid artery and collagen in the wall of factors in the extension of the fibrosis, such as tumour growth factor produced, recovering its hepatic function as well as reducing key activation of stellate cells and secreting cells in the extracellular matrix. The acute inflammation dropped to close to normal values towards the seventh day, while the amount of collagen remained high and the lung volume descended.

The liver growth factor (LGF) is a liver mitogen composed of an albumin-bilirubin complex showing activity either in vivo or in vitro. This factor, purified by our group, was proven in a model of CdCl₂ induced cirrhosis; the LGF markedly decreased the accumulation of extra-cellular matrix components (collagen), restored the serum enzymes, the structural integrity and the necrotic tissue, reduced the abscesses and improved haemodynamics. We have recently proven the anti-fibrotic activity of the LGF in bile duct-ligated rats, in which a marked descent in the accumulation of extracellular matrix was produced, recovering its hepatic function as well as reducing key factors in the extension of the fibrosis, such as tumour growth factor (TGF-β). The LGF anti-fibrotic action is measured by the decrease in activation of stellate cells and secreting cells in the extracellular matrix. Subsequent studies have also proven that the LGF is able to reduce both fibrosis in the carotid artery and collagen in the wall of this artery by 50% after 2 weeks of treatment, causing substantial regeneration of the arterial wall, lowering blood pressure and improving vascular function in a model of spontaneously hypertensive rats. Similarly, the LGF was able to stimulate dopamine terminal sprouting, partially restore motor function in rats with Parkinson’s disease as well as stimulate the generation of new neurons and mobilisation of neurons.

The LGF mitogenic activity in rat liver is measured by the increase in mRNA expression of the tumour necrosis factor (TNF-α), and the main targets of the LGF are the portal endothelial cells, in which a TNF-α expression increase has been observed. Furthermore, the LGF also stimulates TNF-α secretion in endothelial cell cultures, however, it does not cause an increase in the expression of intercellular adhesion molecule 1 (ICAM-1) or vascular adhesion molecule 1 (VCAM-1).

By considering the LGF effect on the fibrosis of other systems, this study proposes a preliminary study on the potential therapeutic effect in a model of lung fibrosis induced with CdCl₂. The administration of this factor, once the fibrosis model is established, displays preliminary data of the LGF therapeutic action, obtains improvements in lung function and partially inverts the deposit of matrix proteins.

Methods

1. Materials and animals: The Animal Experimentation Committee at the Jiménez Díaz-CAPIO Foundation approved all the experiments performed on animals. Forty-two male Wistar rats (220-230g) were administered a single dose via endotracheal induction of saline (control group, n = 21) or 0.5ml/rat of CdCl₂ at 0.025% (n = 21). After instillation, the rats were returned to their cages, where they were administered H₂O and food ad libitum. After 35 days, a group of 15 rats was sacrificed (8 control group and 7 CdCl₂) to confirm the presence of fibrosis. The remaining animals induced with CdCl₂ were divided into 2 new groups and treated with 6 intraperitoneal (i.p.) doses (2 injections/week, for 3 weeks) of saline (CdCl₂ group, n = 7) or 5μg/rat of LGF (LGF group, n = 7). Fifty-five days after induction, we compared the CdCl₂ and LGF groups on a functional, morphometric and biochemical level. Furthermore, a group of control rats was treated with LGF (control group + LGF) and this group was included in the comparisons.

2. Lung function tests: The functional study was performed in a respirator for small animals (Harvard). The rats were anaesthetised with sodium pentothal (60mg/kg of weight, i.p.), they were tracheotomised, placed in a plethysmograph and connected to a cannula to allow communication with the respirator. As soon as the rats were connected to the respirator, they were paralysed with 0.2mg of pancuronium bromide and artificially ventilated. Changes in inspiratory capacity (IC) (ml), lung compliance (LC) (ml/cm of H₂O), forced vital capacity (FVC) (ml) and forced expiratory flow at 75% (FEF75%) (ml/s) were then determined.

The IC was considered as a change in the pulmonary volume when an airway pressure of 30cm H₂O was reached. For the quasi static lung pressure-volume curves, the animals were insufflated with air up to an airflow pressure of 30cm H₂O and exit forced to a constant flow of 1ml/s until a residual volume was reached. Compliance was considered as the highest point in the pressure-volume curve during expiration. In the end, flow-volume curves were induced, the rats were insufflated with air to a pressure of 30cm H₂O and forced expiration provoked using a vacuum pump (Emerson) at a pressure of –40cm H₂O. From these curves, the FVC and the FEF75% of the FVC are determined.

3. Morphometry: After the functional study, the animals were sacrificed with an overdose of pentobarbitone and the cardiopulmonary block was removed. The left lung was weighed and immediately frozen to perform the corresponding biochemical determinations. The right lung was set applying a 10% formaldehyde solution, the lungs were then removed and bisected in two halves. The left half was set applying a 10% formaldehyde solution, and immediately frozen to perform the corresponding biochemical determinations. The right lung was set applying a 10% formaldehyde solution, and immediately frozen to perform the corresponding biochemical determinations.

4. Biochemical analysis of collagen and elastin: Collagen quantification was performed by measuring the hydroxyproline (HYP) residue content. Previously, the left lung was homogenised and chloramine-T was added after acid hydrolysis to induce oxidation. The Erlich (Sigma Aldrich) reagent was then added and once the reaction developed, absorbency at 560nm was assessed.
for each of the samples. The elastin was purified through tissue homogenisation with phosphate buffered saline (PBS) and trichloroacetic acid (TCA) at 10%, centrifugation and treatment with TCA at 5% for 30 min at 90 °C. This last precipitation (purified elastin) was treated with porcine pancreatic elastase type III (Sigma Aldrich) for 4 hours at 25 °C. The elastin was measured with a colorimetric method and bichinonic acid (BCA Protein Assay Kit; Pierce, Promega) was used. HYP (Sigma Aldrich) and bovine neck ligament elastin (Sigma Aldrich) were used as reference standards. Results were expressed in HYP or elastin milligrams for the left lung.

5. Liver growth factor isolation and purification: The LGF was purified of rat serum according to the previously described procedure. The purity and absence of other growth factors or pollutants in the LGF preparation were also performed using standardised criteria. The LGF preparations were freeze-dried and kept at 4 °C until use, when the different aliquots in saline were dissolved for i.p. injection (the LGF is equally active in i.p. and i.v. injection). Before using the LGF in these experiments, its activity was checked in vivo at different doses through injection in normal rats to establish the dose at which greater stimulation of DNA synthesis in the liver is produced and for this the incorporation of tritiated thymidine (New England Nuclear; Dreieich, Germany) was determined in the DNA. In accordance with previous studies, it was considered that the optimum dose of LGF was 0.5 µg/rat.

6. Statistical analysis: All the data expressed as a mean ± standard error of the mean (SEM). Comparisons were performed with ANOVA. Fisher’s least significant difference method was used to analyse the differences between means.

Results

Thirty five days after inducing the lesion (fig. 1, table 1), a significant descent was detected in all the functional variables studied (IC, LC, FVC and FEF75%) with respect to the values obtained in the control group. On the other hand, a greater increase, also significant, was observed in the protein content of the extracellular matrix, usually found in pulmonary fibrosis, as well as a marked increase in the morphometric variables (AIA and LM) which recall the typical enlargement of the airways of emphysema.

To confirm that the LGF does not present negative effects in the healthy animals, 7 rats in the control group were administered the same treatment as the LGF group. No differences were found in relation to the control group (table 2). Neither were differences found in any of the parameters studied (table 3) between days 35 and 55 within the group of rats to which CdCl2 had been administered, which confirms that the induced lesion was stable and permanent in the period where treatment with LGF was applied.

1. Pulmonary function: The changes in IC, LC, FVC and FEF75% in the study groups are shown in figure 2. As previously mentioned, the pulmonary fibrosis induced with CdCl2 produced a significant descent in IC, LC, FVC and FEF75% in comparison with the control rats. The administration of LGF brought all the functional variables to values near normality, with statistically significant variations in IC, FVC and FEF75%, but not in LC (LGF versus CdCl2).

2. Pulmonary collagen and elastin. The CdCl2 caused a significant increase in the collagen and elastin contents in the CdCl2 group compared to the healthy rats. After the LGF treatment, the lung content in collagen was less than in the rats that received CdCl2, and this difference was important when assessed in terms of HYP residues (fig. 3). We also detected a notable descent in the pulmonary elastin content in the LGF group in comparison to the rats that received CdCl2 (fig. 3).

Figure 4 shows an increase in the collagen deposit, analysed with birefringence in the collagen-rich areas of the Sirius red staining. The LGF partially inverted the lung matrix deposits, with a tendency to reach normal values, but with no statistical significance.

3. Morphometry: The morphometric analysis of the LM and AIA display a significant increase in these variables in the CdCl2 group versus the healthy controls. After treatment with LGF, a slight reduction in the LM and AIA was observed, but with no statistical importance (fig. 5). Figure 6 displays microscope images of a healthy lung and another instilled with CdCl2, where the increase in alveolar size and the peribronchial fibrosis present in the affected group are evident.

Discussion

In this article, we show that the model of CdCl2 induced lung lesion has a fibrotic component characterised by the decrease of the functional variables as well as an increase in the matrix protein deposits and also, an emphysematous component due to the existence of enlarged alveoli observed in the morphometric analysis. Based on this model, established 35 days after the CdCl2

<table>
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<tr>
<th>Table 1</th>
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<tr>
<th>Functional, morphometric and biochemical data 35 days after inducing the lesion*</th>
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<tr>
<td>IC, ml</td>
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<td>-----</td>
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<tr>
<td>C</td>
</tr>
<tr>
<td>CdCl2</td>
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AIA: alveolar internal area; CdCl2: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H2O: water; HYP: hydroxyproline; LM: mean linear intersection.

*p < 0.05 versus control.
instillation, treatment with LGF partially inverts the induced fibrotic lesion. This statement is supported by the improvement in most of the functional variables (IC, FVC and FEF75%) and the notable decrease in the lung matrix proteins (HYP and elastin) after the LGF treatment. The rats instilled with CdCl$_2$ displayed a descent in the functional variables and an increase in the lung matrix protein levels similar at 35 and 55 days after instillation, so we can consider the lesion caused by the CdCl$_2$ as permanent and, therefore, the effects produced by the LGF as therapeutic. To the best of our knowledge, this is the first experimental study in which an antifibrotic treatment partially restores normal lung function of a previously established lesion.

Several authors have described that CdCl$_2$ induced pulmonary fibrosis is characterised by the functional damage, including a descent in the vital capacity, compliance and forced expiratory flow as well as an increase in the lung matrix protein content$^{48,21}$ and

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Table 2
Comparison between the control group and the control group + liver growth factor in every variable

<table>
<thead>
<tr>
<th></th>
<th>IC, ml</th>
<th>LC, ml/cm H$_2$O</th>
<th>FVC, ml</th>
<th>FEF75%, ml/s</th>
<th>HYP, mg/lung</th>
<th>Elastin, ml/lung</th>
<th>AIA, µm$_2$</th>
<th>LM, µm</th>
<th>Sirius red, birefringence/total tissue, %</th>
</tr>
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<tbody>
<tr>
<td>CdCl$_2$, 35 days</td>
<td>11.5 ± 0.7</td>
<td>0.7 ± 0.09</td>
<td>11.1 ± 0.7</td>
<td>33.4 ± 6</td>
<td>3.0 ± 0.2</td>
<td>19.4 ± 2.6</td>
<td>9,616 ± 842</td>
<td>68 ± 3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>CdCl$_2$, 55 days</td>
<td>11.8 ± 0.5</td>
<td>0.7 ± 0.06</td>
<td>11.5 ± 0.6</td>
<td>35.4 ± 4.2</td>
<td>3.8 ± 0.2</td>
<td>21.6 ± 1.2</td>
<td>1,0931 ± 1,056</td>
<td>69 ± 2</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

No statistical differences are observed between the groups.

AIA: alveolar internal area; CdCl$_2$: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H$_2$O: water; HYP: hydroxyproline; LM: mean linear intersection.

Table 3
Comparison of the functional, morphometric and biochemical data 35 and 55 days after the administration of cadmium chloride

<table>
<thead>
<tr>
<th></th>
<th>IC, ml</th>
<th>LC, ml/cm H$_2$O</th>
<th>FVC, ml</th>
<th>FEF75%, ml/s</th>
<th>HYP, mg/lung</th>
<th>Elastin, ml/lung</th>
<th>AIA, µm$_2$</th>
<th>LM, µm</th>
<th>Sirius red, birefringence/total tissue, %</th>
</tr>
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<tr>
<td>C</td>
<td>16.5 ± 0.5</td>
<td>1.2 ± 0.06</td>
<td>16.7 ± 0.63</td>
<td>77.4 ± 4.6</td>
<td>1.4 ± 0.2</td>
<td>12.3 ± 1.3</td>
<td>3,981 ± 1,141</td>
<td>46 ± 2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>C+LGF</td>
<td>16.8 ± 0.5</td>
<td>1.31 ± 0.06</td>
<td>16.8 ± 0.5</td>
<td>79.8 ± 6.4</td>
<td>1.6 ± 0.3</td>
<td>13.8 ± 1</td>
<td>4,154 ± 551</td>
<td>47 ± 2</td>
<td>1.9 ± 0.2</td>
</tr>
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</table>

No significant differences were found between the groups.

AIA: alveolar internal area; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H$_2$O: water; HYP: hydroxyproline; LGF: liver growth factor; LM: mean linear intersection.

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Figure 2. Changes in the functional variables in all the experimental groups. Control group (n = 6), CdCl$_2$ group (n = 7) and LGF group (n = 7). CdCl$_2$: cadmium chloride; IC: inspiratory capacity; LC: lung compliance; FVC: forced vital capacity; FEF75%: forced expiratory flow at 75%; H$_2$O: water; HYP: hydroxyproline; LGF: liver growth factor. a$p < 0.05$ versus control. b$p < 0.05$ versus CdCl$_2$. 
centriacinar emphysema appears. Even though the LGF does not reduce alveolar enlargement caused by the CdCl$_2$ instillation in any significant manner, although there is a tendency of improvement that could open a new line of study on the track towards the effect of this growth factor in the emphysema.

This study has many similarities to the prior one carried out by Heili Frades et al. In both cases, similar lung lesions have been found that affect the function, alveolar size and extracellular matrix protein levels compatible with centriacinar emphysema and peribronchial fibrosis; in this model the treatment with N-acetylcysteine (NAC) partially improved the pulmonary function deterioration, observed in all the functional variables measured (IC, FVC and FEF75%), except in the LC. The same occurs with the collagen deposit measured by the HYP residues, elevated due to the CdCl$_2$ induction and partially restored by the NAC treatment. The response pattern observed with the CdCl$_2$ is very similar to this study, including the responses to treatment. The NAC improves the fibrotic lesion, though the difference lies in the administration method; while this antioxidant agent is administered in a concomitant manner to the induction of the lesion, the LGF is administered once this has been established and stabilised.

The case of NAC is not the only one in medical literature due to the prevalence of this disease and the difficulty of finding effective therapeutic targets to fight it, makes the search for treatment continuous. There are many studies carried out in bleomycin models with antifibrotic agents, such as deferoxamine, the hepatocyte growth factor, the keratinocyte growth factor, interferon and gingko biloba, EM703, IMD-0354 and C-type natriuretic peptide, and even studies that approach another type of treatment such as cellular treatment based on the instillation of alveolar type II cells as a regeneration vehicle of damaged tissue. Although these agents have the capacity of reducing fibrosis, they also have the limitation that the antifibrotic agent is added concomitantly to the development of the disease, which hinders its therapeutic use since the condition is diagnosed, in the majority of cases, once it has been established.

According to previous publication, the cellular inflammation is not a key factor after the first days of CdCl$_2$ instillation. Since the LGF was administered once the lesion was established, it is unlikely that the LGF activity is due to an anti-inflammatory effect and partially inverts the fibrosis, however, we observe that the LGF has a considerable free radical abudaction activity. Our group recently demonstrated that the antifibrotic capacity of LGF is measured by the partial inhibition of the TGF-β, and the transformation of...
myofibroblasts,13,14 both key in the process of extracellular matrix deposit. However, it is also possible that the LGF chooses mother cells and introduces the proliferation of the endothelial and alveolar cells, as other growth factors do.27–35 Altogether, these observations indicate the need to carry out new studies to determine the action mechanism of the LGF in the repair of lung fibrosis. To conclude, this study shows that LGF treatment administered on a lung fibrosis previously established through the administration of CICI, was able to partially invert the pulmonary fibrosis, improve lung function and invert the increase in lung matrix proteins. Pending determination of the action mechanism that facilitates this response, the possibility of inverting a previously established pulmonary fibrosis opens the possibility that LGF can be applied in the future to pulmonary fibrosis treatment.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Funding

Funded by the Spanish Health Research Fund (PI050720) and Spanish Society of Pneumology and Thoracic Surgery.

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