


Cyclooxygenase-2 Overexpression, Using an Integrin-Targeted Gene Delivery System (the LID Vector), Inhibits Fibroblast Proliferation In Vitro and Leads to Increased Prostaglandin E$_2$ in the Lung*

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Abbreviations: BALF = BAL fluid; COX = cyclooxygenase; IPF = idiopathic pulmonary fibrosis; LID = lipofectin, integrin-targeting peptide, and plasmid DNA; PBS = phosphate-buffered saline solution; PGE$_2$ = prostaglandin E$_2$

Pulmonary fibrosis is a progressive and fatal disease for which there is no effective treatment. It is characterized by increased matrix deposition following an apparent, or subclinical, lung injury. The fibrogenic process often is

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preceded by inflammation, which is best characterized in animal models. In this process, prostaglandin E₂ (PGE₂) is the major prostanoid secreted by both fibroblasts and stimulated inflammatory cells. Cyclooxygenase (COX) is the rate-limiting enzyme in prostanoid biosynthesis. There are two isoforms. COX-1 is the constitutive isoform, which is thought to be responsible for housekeeping functions, and COX-2 is the inducible isoform, which is known to be up-regulated early in acute inflammatory conditions. However, little attention has been paid to understanding the mechanisms by which proinflammatory and profibrotic responses are inhibited during injury and terminated following injury, thus preventing the development of fibrosis.

The role of PGE₂, and the isoform responsible for its generation, in the evolution of the fibrogenic process is incompletely understood. Levels of PGE₂ are lower in BAL fluid (BALF), and less is produced by macrophages that are taken from people with idiopathic pulmonary fibrosis (IPF) by BAL compared with control subjects. Furthermore, fibroblasts from patients with IPF have a reduced capacity to synthesize PGE₂ in response to profibrotic mediators such as interleukin-1β and transforming growth factor-β, and this has been related to a decreased capacity to up-regulate COX-2.¹² Furthermore, the failure to up-regulate COX-2 in response to transforming growth factor-β is associated with enhanced fibroblast proliferation and collagen synthesis, which are key features in the development of pulmonary fibrosis.¹³ Therefore, overexpression of the COX-2 gene may offer a potential treatment for patients with IPF.

In order for COX-2 gene therapy to be an effective treatment, high-efficiency systems with minimal toxicity will need to be developed. We have developed a nonviral gene delivery system that is composed of lipofectin, an integrin-targeting peptide, and plasmid DNA (LID vector) and that has a transfection efficiency in the lung that rivals an adenovirus and is greater than that of 1,2-dioleoyl-3-trimethylammonium-propane.³ The aim of this study was to optimize gene delivery in order to generate high levels of PGE₂ in the lung following pulmonary gene transfer.

**Materials and Methods**

The LID vector is formed by complexing lipofectin, an α5β1-specific peptide, and DNA in an optimized weight ratio of 0.75:1, as previously described.³ Complexes were either in phosphate-buffered saline solution (PBS) or water. LID vector containing 5 μg plasmid DNA in 50 μL instillate was delivered via intratracheal injection into the lungs of 6-week-old C57Bl/6 mice. At least six animals per treatment group were used in all experiments. Plasmids encoding the luciferase gene were used to determine the optimal levels of gene expression in the lung, and luciferase activity was measured in whole-lung lysates 1 day following transfection. Luciferase activity is not normally distributed, therefore a Mann-Whitney U test was used to assess the differences between groups following transfection with luciferase. Plasmids encoding human COX-2 (pCOX-2 was a gift from T. Hla) were used to generate PGE₂ from human lung fibroblasts and plasmids encoding murine COX-2 (pTIS10 was a gift from H. Herschman) were used to generate PGE₂ in mouse lungs. For in vitro studies, human lung fibroblasts were grown to subconfluence and were transfected with the LID vector in optimem. Following serum rescue, the cells were cultured serum-free for 24 h (40 h following transfection), and the PGE₂ in the cell media was assayed using an enzyme immune assay. At least four wells per treatment group were analyzed, and the experiment was repeated three times. For in vivo studies, the LID vector was generated with the COX-2 plasmid in water and the mice were transfected as described above for luciferase transfections. Mice were killed 48 h following transfection, and their lungs were lavaged with 10 aliquots of 500 μL PBS. The lavage fluid was centrifuged at 150g for 5 min at 4°C, and the supernatant was assayed for PGE₂ using an enzyme immune assay. The cellular component was resuspended in 100 ng/mL albumin in PBS, and the total cell number was determined by cell counting using a hemocytometer. A Student’s t test was used to assess differences between PGE₂ levels and cell number.

**Results**

Luciferase activity following instillation of LID/H₂O was >10-fold greater than in isotonic PBS. The median luciferase activity was 740 RLU/mg protein following transfection using LID/H₂O (range, 105 to 2,590 RLU/mg) compared with 70 RLU/mg protein (range, 16 to 106 RLU/mg) following transfection with LID/PBS (p < 0.005). An assessment of the physicochemical structure of the complex suggests that water enhances transfection by making the complex smaller and more stable than when prepared in PBS. The results of transfecting COX-2, using the LID vector, in human lung fibroblasts...
are shown in Figure 1. COX-2 overexpression increased fibroblast PGE\(_2\) synthesis fourfold and inhibited fibroblast proliferation by 63\% compared with control transfections, using a plasmid containing the COX-2 plasmid in the antisense orientation, and untransfected controls. Interestingly, control transfection gave a sevenfold increase in PGE\(_2\) level compared with untransfected controls and an accompanying inhibition of fibroblast proliferation. When the LID vector was used to overexpress COX-2 in the mouse lung it led to a mean (± SD) 20-fold increase in the BALF PGE\(_2\) level compared with control transfection, using the luciferase gene (1,050 ± 350 pg/mL BALF vs 50 ± 5 pg/mL BALF, respectively; p < 0.05). Furthermore, this was associated with a reduction in the total BAL cell number in COX-2-transfected mouse lungs compared with control-transfected mouse lungs (17.3 ± 2.1 × 10⁴ vs 23.8 ± 2.2 × 10⁴ cells, respectively; p < 0.05).

**Discussion**

Pulmonary fibrosis is a devastating disease with no proven or effective therapy. We have previously demonstrated that a deficiency in COX-2 gene expression may predispose people to develop this condition.\(^3\) Therefore, using gene therapy to overexpress COX-2 in the lungs may offer a potential treatment for pulmonary fibrosis. For gene therapy to be successful, efficient gene delivery systems with high levels of plasmid expression and minimal toxicity are required. We have previously demonstrated that the LID vector can transflect the lungs of rodents with an efficiency that rivals that of an adenovirus, but without significant inflammation even after repeated administration.\(^4\) In this study, we show that making the LID vector in a hypotonic buffer leads to even greater gene expression following pulmonary transfection. This may be due to the effect of water on the structural parameters of the complex. Varying the buffers is known to alter the structure of similar nonviral delivery systems,\(^4\) although the effect that this has on gene expression has not been assessed following transfection in the lung.

Using the LID vector to overexpress COX-2 *in vitro* leads to an increase in PGE\(_2\) production by fibroblasts, and this inhibits fibroblast proliferation. This is consistent with previous data showing that exogenous PGE\(_2\) inhibits fibroblast proliferation *in vitro*.\(^5\) It is interesting to note that transfection using the control plasmid also generates PGE\(_2\) in human lung fibroblasts. This may reflect up-regulation of the endogenous COX-2 gene by the process of transfection, but this was not observed *in vitro*. Transfection of the lung with COX-2 increased the levels of PGE\(_2\) with a consequent reduction in total lavage cell numbers 48 h following transfection, whereas control transfection did not alter either parameter. It is tempting to speculate that the overexpression of COX-2 is acting in some way to inhibit inflammatory cell recruitment or survival within the lung, and it has previously been suggested that COX-2 might be vital for terminating the inflammatory response.\(^6\) However, the mechanism by which COX-2 leads to the resolution of inflammation is unknown and may not relate to the production of PGE\(_2\).\(^6\) Furthermore, the BALF cell number may not accurately reflect total inflammatory cell populations within the lung, and further work is needed to clarify this point. However, we can conclude that the overexpression of COX-2 in the lung leads to increased levels of PGE\(_2\) that are similar to those following an inflammatory stimulus,\(^7\) suggesting functional levels of gene expression. Therefore, COX-2 gene therapy using the LID vector may have potential as a treatment for fibrotic lung disease.

**References**


**Gene Expression Profiles in Pulmonary Hypertension**

Mark W. Geraci, MD; Yasushi Hoshikawa, MD; Michael Yeager; Heiko Golpon, MD; Tracy Gesell; Rubin M. Tuder, MD; and Norbert F. Voelkel, MD

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Abbreviation: PPH = primary pulmonary hypertension

Chronic pulmonary hypertension is associated with structural alterations of the large and small pulmonary arteries in which smooth muscle and endothelial cells interact in a process of vascular "remodeling." Because the vascular lesions are ubiquitously distributed in the lung, random lung tissue samples can be used for microarray

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