Inhibition of Matrix Metalloproteinases by Lung TIMP-1 Gene Transfer or Doxycycline Aggravates Pulmonary Hypertension in Rats

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Abstract—Chronic hypoxic pulmonary hypertension (PH) results from persistent vasoconstriction, excess muscularization, and extracellular matrix remodeling of pulmonary arteries. The matrix metalloproteinases (MMPs) are a family of proteases implicated in extracellular matrix turnover and hence in smooth muscle and endothelial cell migration and proliferation. Because MMP expression and activity are increased in PH, we designed the present study to investigate whether inhibition of lung MMPs in rats subjected to chronic hypoxia (CH) contributes to or protects against vascular remodeling and PH. To achieve lung MMP inhibition, rats exposed to 10% O₂ for 15 days were treated with either doxycycline (20 mg/kg per day by gavage starting 2 days before and continuing throughout the CH period) or a single dose of recombinant adenovirus (Ad) for the human tissue inhibitors of metalloproteinases-1 (hTIMP-1) gene (Ad.hTIMP-1, 10⁸ plaque-forming units given intratracheally 2 days before CH initiation). Control groups either received no treatment or were treated with an adenovirus containing no gene in the expression cassette (Ad.Null). Efficacy of hTIMP-1 gene transfer was assessed both by ELISA on bronchoalveolar lavages and by hTIMP-1 immunofluorescence on lung sections. MMP inhibition in lungs was evaluated by in situ zymography and gelatinolytic activity assessment using [³H]gelatin. Rats treated with either doxycycline or Ad.hTIMP-1 had higher pulmonary artery pressure and right heart ventricular hypertrophy more severe than their respective controls. Worsening of PH was associated with increased muscularization and periadventitial collagen accumulation in distal arteries. In conclusion, our study provides compelling evidence that MMPs play a pivotal role in protecting against pulmonary artery remodeling. (Circ Res. 2000;87:418-425.)

Key Words: pulmonary hypertension • gene therapy • inhibitor of metalloproteinases • extracellular matrix • vascular remodeling

Chronic pulmonary hypertension (PH) results from persistent vasoconstriction and structural remodeling of pulmonary arteries.¹ ² Accumulation of connective tissue in vessel wall is an important component of the alterations in PH and results from a complex interplay between synthesis and degradation of extracellular matrix (ECM).³ ⁵ ECM protein degradation is an active process dependent on the action of proteases, including matrix metalloproteinases (MMPs). MMPs are identified and subdivided on the basis of their substrate specificity into collagenases, gelatinases, stromelysin, and membrane-type MMPs.⁶ The importance of MMPs in the development of PH remains unclear. MMPs not only control ECM deposition, but they also facilitate migration and growth of smooth muscle cells (SMCs)⁷ through their ability to degrade ECM. Mitogenesis of SMCs may also be indirectly stimulated by MMPs through the release of growth factors bound to ECM proteins.⁸

In rats, reversal of hypoxic PH after return to normoxia is associated with increases in collagenolytic and elastolytic activities, with a rapid reduction in collagen content of pulmonary arteries, suggesting an association between decreased pulmonary artery remodeling and increased MMP activity.⁵ ⁹ However, gelatinase A (MMP-2) expression and activity may be increased in pulmonary arteries from rats developing PH in response to monocrotaline or to chronic hypoxia.¹⁰ ¹¹ This suggests that MMPs may contribute to pulmonary artery remodeling, an effect that would be consistent with reported effects of MMPs on systemic arteries¹² ¹⁴ in which MMP inhibition limited neointima formation.¹⁵ In PH, the effects of MMP inhibition have not yet been studied...
in vivo, particularly because MMP inhibition has proven difficult to achieve in the lung. MMP activity is modulated by counterregulatory tissue inhibitors of MMPs (TIMPs). TIMP-1 is synthesized by most types of connective tissue cells and inhibits collagenases, stromelysin, and gelatinases. We used adenovirus (Ad)-mediated gene transfer to induce overexpression of human TIMP-1 (hTIMP-1) in the lungs with the goal of investigating the effect of chronic MMP inhibition on the development of PH and vascular remodeling in rats subjected to chronic hypoxia. To confirm our results obtained with Ad.hTIMP-1, we also used the same PH model to examine the effect of doxycycline, which has been shown to potently inhibit MMP activity when administered in vivo.

**Materials and Methods**

**Study Design**

Two sets of experiments were performed in Wistar rats (260 to 280 g body weight, Charles River) exposed to chronic hypoxia. In the doxycycline study, rats received 20 mg/kg doxycycline by gavage per day and were compared with an untreated control group. Doxycycline was started 2 days before hypoxia and continued throughout hypoxia exposure. In the Ad.hTIMP-1 study, rats were treated 2 days before hypoxia with a single intratracheal dose of either a recombinant adenovirus coding for hTIMP-1 (Ad.hTIMP-1, 10^8 plaque-forming units [pfu]) or an adenovirus with an empty expression cassette (Ad.Null, 10^8 pfu).

A preliminary study in normoxic conditions was conducted to assess efficiency of gene transfer to the lung after intratracheal Ad.hTIMP-1 instillation.

**Treatment With Ad.hTIMP-1**

Ad.hTIMP-1 is a replication-deficient recombinant adenovirus serotype 5 vector capable of high-level hTIMP-1 gene expression. Ad.Null was used as the control. Intratracheal instillation of 150 μL/rat of diluted Ad.hTIMP-1 or Ad.Null was performed as previously described.

Evaluation of Gene Transfer Under Normoxic Conditions. Bronchoalveolar lavage (BAL) fluid levels of hTIMP-1 protein were measured 5 days after administration of Ad.hTIMP-1 (5×10^7 or 10^8 pfu) or Ad.Null (10^7 pfu). hTIMP-1 protein was also assayed in BAL fluid (n=3 rats), 5 (n=10), and 15 days (n=8) after an Ad.hTIMP-1 dose (10^8 pfu).

ELISA detection of hTIMP-1 was carried out on BAL samples (TIMP-1, human ELISA system, Amersham Pharmacia Biotech). The ELISA system used selectively measures hTIMP-1 and does not cross-react with rat TIMP-1.

Immunocytochemistry was performed on the lungs of normoxic rats 5 days after instillation of Ad.hTIMP-1 or Ad.Null (10^8 pfu). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (TEBU, Le Perray en Yvelines, France), with the exception of nonrelevant rabbit antibodies to human thyroglobulin that were used as the negative control (Dako).

**Exposure of Rats to Chronic Hypoxia and Evaluation of PH and Gelatinase Activity**

Rats were exposed to chronic hypoxia (10% to 12% fraction of inspired oxygen) for 15 days, as previously described.

**Hemodynamic Measurements and Assessment of Right Ventricular Hypertrophy**

At the end of exposure to hypoxia, pulmonary and systemic arterial pressures were measured, as previously described. Finally, the heart was excised and weighed, and the Fulton index (ratio of right ventricular free wall over sum of septum plus left ventricular free wall weight [RV/S+LV]) was calculated.

**Assessment of Pulmonary Vascular Remodeling**

Five-micrometer-thick lung sections were stained with hematoxylin-phloxin-saffron. In each rat, 30 to 40 intra-acinar arteries were analyzed to assess the distribution and degree of muscularization. Intra-acinar arteries were categorized as muscular, partially muscular, or nonmuscular, as reported.

Five-micrometer-thick lung sections were also stained with orcein-picroindigo-carmine to measure collagen content. Quantification of the collagen in pulmonary arteries was assessed morphometrically with an image analysis system using Quanacol software (Quan’ Image), as previously described.

**Gelatinase Activity**

The pulmonary arteries and lung were excised, frozen in liquid nitrogen, and stored at −80°C for gelatin zymography on homogenates, as previously described. They were also fixed in the distended state by infusion of Tissue-Tek OCT compound (Sakura) and stored at −80°C for in situ zymography. Free gelatinases activities were also assayed by using the ability of tissue homogenates to degrade 50 μg of [3H]gelatin.

**Statistical Analysis**

All results are reported as mean±SEM. The nonparametric Mann-Whitney U test was used for single comparisons of hemodynamic parameters, body weight, heart weight, degree of muscularization, and collagen content between inhibitor-treated and control rats.

**Results**

**Ad.hTIMP-1 Treatment**

Evaluation of In Vivo hTIMP-1 Gene Transfer and Endogenous Gelatinase Activity in Normoxic Rats

As little as 5×10^7 pfu of Ad.hTIMP-1 induced expression of hTIMP-1 protein detectable in BAL fluid (Figure 1A). No hTIMP-1 was found in BAL fluid from the control rats given Ad.Null. The level of hTIMP-1 in BAL fluid increased with the dose of Ad.hTIMP-1. With 10^8 pfu of either Ad.hTIMP-1 or Ad.Null, mild inflammation characterized by patchy infiltrates of mononuclear cells was seen, with no cell damage, edema, or hemorrhage (data not shown). Because the 10^8-pfu dose caused minimal inflammation and significant hTIMP-1 protein production in the lung, we used it in further experiments.

As shown in Figure 1B, detectable hTIMP-1 protein expression in BAL fluid after administration of 10^8 pfu Ad.hTIMP-1 was noted on day 2 after dose and was still present 5 and 15 days after dose.

Immunolocalization of hTIMP-1 on lung sections from Ad.hTIMP-1–treated rats demonstrated expression of hTIMP-1 by bronchoalveolar epithelial cells (Figure 2B). Staining for hTIMP-1 protein was diffuse throughout the lung, albeit heterogeneous. No fluorescence was detectable on lung sections of Ad.Null-treated rats (Figure 2A).

To ascertain that Ad.hTIMP-1 administration did not alter endogenous gelatinase production, BAL fluid gelatinase levels were measured using gelatin zymography. Total gelatinase activity in BAL fluid did not differ among Ad.hTIMP-1–, Ad.Null–, and vehicle-treated rats (data not shown).

**Effects of Ad.hTIMP-1 on Chronic Hypoxic PH**

Ad.hTIMP-1 and Ad.Null (10^8 pfu) were well tolerated. No deaths or symptoms of respiratory failure were observed in the rats exposed to chronic hypoxia.
Hemodynamic Measurements and Assessment of Right Ventricular Hypertrophy After Exposure to Chronic Hypoxia

After 15 days of exposure to hypoxia, although there was a trend toward higher pulmonary artery pressure in the Ad.hTIMP-1–treated than in the Ad.Null-treated rats (Figure 3A), the difference did not reach statistical significance (P = 0.06). Body weight, systemic arterial pressure, and heart rate were similar in both groups (data not shown). Right ventricular hypertrophy assessed by the Fulton index was significantly more marked in the Ad.hTIMP-1–treated rats than in the Ad.Null-treated rats (P < 0.01, Figure 3B), whereas the ratio of left ventricular plus septum weight divided by body weight was similar in the 2 groups (data not shown).

Structural Remodeling of Distal Pulmonary Arteries

Treatment with Ad.hTIMP-1 was associated with a significant increase in distal pulmonary artery muscularization (Figure 4) compared with the control exposed to similar hypoxic conditions (P < 0.001).

Collagen content was significantly increased in pulmonary arteries from rats treated with Ad.hTIMP-1 compared with control (P < 0.0001, Figure 4B). This increase was localized in the periadventitial compartment of the vessels (Figure 4C).

Evaluation of Gelatinase Activity

Gelatinase activity as evaluated by in situ zymography was dramatically decreased in lung from rats treated with Ad.hTIMP-1 compared with controls (Figure 5B). There was a decrease in free gelatinolytic activity (degradation of [3H]gelatin) found in lung and pulmonary arteries from rats treated by Ad.hTIMP-1 compared with Ad.Null-treated rats, although this was not significant (Figure 5A).

Total gelatinase activity in lungs (Figure 1 online, available in online-only data supplement at http://www.circresaha.org)
and in pulmonary artery homogenates (data not shown), as measured by conventional zymographic analysis SDS-PAGE to separate MMPs from their inhibitors, was similar in rats treated with Ad.hTIMP-1 and Ad.Null.

Doxycycline Treatment

Effects of Doxycycline Treatment on Chronic Hypoxic PH

Hemodynamic Measurements and Assessment of Right Ventricular Hypertrophy After Exposure to Chronic Hypoxia

After 15 days of exposure to hypoxia, pulmonary artery pressure was higher in the doxycycline-treated rats than in their respective controls (P<0.05, Figure 6A). Body weight, systemic arterial pressure, and heart rate were similar in all groups (data not shown). However, the Fulton index was not significantly increased in the doxycycline-treated rats compared with their controls (Figure 6B). The ratios of heart weight to left ventricular plus septum weight divided by body weight were significantly lower in the doxycycline-treated rats than in the control rats (2.42±0.08 versus 2.67±0.07 and 1.71±0.05 versus 1.87±0.05, respectively; P<0.05), whereas

Figure 3. Pulmonary artery pressure and right ventricular hypertrophy in Ad.hTIMP-1 study. A, Mean pulmonary artery pressure in rats exposed to chronic hypoxia and treated with 10⁸ pfu Ad.hTIMP-1 (n=10). Corresponding control group was exposed to chronic hypoxia and received 10⁸ pfu Ad.Null (n=10). B, Right ventricular hypertrophy as assessed on the basis of the Fulton index (RV/S+LV) in rats exposed to chronic hypoxia and treated with 10⁸ pfu Ad.hTIMP-1 (n=10). The corresponding control group was exposed to chronic hypoxia and received 10⁸ pfu Ad.Null (n=10). Values are mean±SEM. **P<0.05.

Figure 4. A, Muscularization of pulmonary peripheral vessels in Ad.hTIMP-1 study. A significant increase in muscularization of small pulmonary arteries was found in Ad.hTIMP-1–treated rats (n=10) compared with Ad.Null–treated rats (n=10). Percentages of nonmuscular (NM), partially muscular (PM), and muscular (M) arteries were determined at the alveolar duct and alveolar wall levels. Values are mean±SEM. **P<0.05. B, Morphometric quantification of collagen content in pulmonary arteries by an image analysis system using Quancoul software on lung sections stained with orcein-picroindigo-carmine. Collagen accumulation was increased in pulmonary arteries from rats treated with Ad.hTIMP-1 (n=10) compared with control (n=10). Values are mean±SEM percentages for collagen in pulmonary arteries. **P<0.05. C, Periadventitial collagen accumulation in pulmonary arteries in rats exposed to chronic hypoxia and treated with either Ad.Null (top) or Ad.hTIMP-1 (bottom). Lung sections stained with orcein-picroindigo-carmine from the Ad.hTIMP-1–treated rat shows periadventitial collagen accumulation (in blue) in pulmonary arteries. Magnification ×250.
right ventricular weight divided by body weight was not significantly different between the 2 groups (0.71 ± 0.04 versus 0.80 ± 0.04).

Structural Remodeling of Distal Pulmonary Arteries
Treatment with doxycycline was associated with a significant increase in distal pulmonary artery muscularization compared with the controls (P < 0.001, Figure 7A).

Collagen content was significantly increased in pulmonary arteries from rats treated with doxycycline compared with control (P < 0.05, Figure 7B). This increase was localized in the periadventitial compartment of the vessels (Figure 7C).

Evaluation of Gelatinase Activity
Gelatinase activity as evaluated by in situ zymography was decreased in pulmonary arteries (Figure 8B) and in lung sections (data not shown) from rats treated with doxycycline. There was a decrease in free gelatinolytic activity (degradation of [1H]gelatin) found in pulmonary arteries from rats treated by doxycycline compared with control rats, although this was not significant (Figure 8A).

Total gelatinase activity in lungs (Figure 1 online, available in online-only data supplement at http://www.circresaha.org) and in pulmonary artery homogenates (data not shown), as measured by conventional zymographic analysis SDS-PAGE, was similar in rats treated with doxycycline and in their controls.

Discussion
We found that inhibition of lung MMPs during exposure to chronic hypoxia in rats was associated with exacerbation of PH and vascular remodeling. Either of 2 MMP-inhibiting treatments, namely adenovirus-mediated hTIMP-1 lung gene transfer and doxycycline, increased muscularization and collagen accumulation in small pulmonary arteries. These results support a key role of MMPs in limiting ECM and smooth muscle accumulation during the development of PH.

Previous observations have suggested that ECM alterations produced by proteolytic enzymes contribute to the process of vascular remodeling. In experimental models of systemic vascular injury induced by endothelial denudation, MMP inhibition has been found to delay or diminish neointima formation and arterial wall thickening. In pulmonary arteries, increases in MMP expression and activity have been demonstrated during both the development and the revers...
The observation that pulmonary arteries undergo active remodeling during PH development has suggested that developing pharmacological inhibitors of this remodeling process may be an effective treatment strategy. However, few data are available on the role of MMPs in PH, in part because no pharmacological tools capable of inhibiting MMP activity selectively in the lung were available.

In the present study, we used a previously described adenovirus vector containing hTIMP-1 cDNA, which we administered intratracheally to rats before exposure to hypoxia. Several studies using intratracheal administration of adenovirus vectors found that transgene expression was mainly located within airway and alveolar epithelial cells. On the basis of earlier results with this route of administration, we reasoned that adenovirus-mediated gene transfer of the hTIMP-1 gene to the lung would expose pulmonary arteries to increased levels of TIMP-1. After intratracheal administration of Ad.hTIMP-1 (10⁸ pfu), the hTIMP-1 protein was expressed by bronchoalveolar epithelial cells throughout the lung sections. Our investigation of hTIMP-1 protein levels in BAL fluid showed that hTIMP-1 overexpression persisted throughout the 15 days of exposure to hypoxia. The ability of hTIMP-1 to inhibit MMP activity was confirmed by our in situ zymography results showing a dramatic decrease in free gelatinase activity in the pulmonary arteries and lungs from Ad.hTIMP-1–treated rats compared to controls.
increases in arteriole wall thickness and decreases in arteriole hydroxyproline reduced hypoxia-induced PH and prevented role of MMPs in systemic artery remodeling. The apparent in TIMP-1– deficient mice further supports an aggravating more severe neointimal formation after femoral artery injury. Other studies found evidence that pulmonary artery wall thickening during the development of PH was related in part to increased connective tissue protein synthesis, a possibility consistent with our finding that both lung hTIMP-1 overexpression and doxycycline were associated with excess periadventitial collagen in small pulmonary arteries. Also, Kerr et al reported that administration of the collagen synthesis inhibitor cis-hydroxyproline reduced hypoxia-induced PH and prevented increases in arteriole wall thickness and decreases in arteriole lumen diameter. Moreover, Thakker-Varia et al reported that the activity of collagenases found predominantly in the media and adventitia contributed to the breakdown of collagen in pulmonary artery walls observed during early reversal of hypoxic PH.

MMP inhibition was associated not only with greater ECM accumulation in artery walls, but also with increased muscularization of intra-acinar arteries. This finding was somewhat unexpected, because several studies have suggested that ECM degradation may be essential to SMC migration and proliferation. Moreover, synthetic MMP inhibitors have been shown to inhibit proliferation of SMCs from rabbit aorta explants. Localized TIMP overexpression in luminal cells transduced with either the same recombinant adenovirus as that used in the present study or with TIMP-2 adenovirus potently inhibited neointima formation in human saphenous veins in an organ culture model. The recent finding of more severe neointimal formation after femoral artery injury in TIMP-1–deficient mice further supports an aggravating role of MMPs in systemic artery remodeling. The apparent discrepancy between these studies and our results may be ascribable to differences across the considered vascular beds. In particular, neointima formation in pulmonary arteries occurs only when the pressure reaches systemic levels. Increased pulmonary artery muscularization in response to MMP inhibition may be a direct consequence of increased ECM deposition; an increase in the number of cell-ECM contacts may stimulate SMC growth by increasing the mechanical tension transmitted across integrins. Alternatively, the increased muscularization may be an adaptive response to the increased resistance to flow produced by ECM accumulation. This latter hypothesis is supported by a report that SMC proliferation increased in response to a rise in pulmonary artery wall tension.

Our results might contrast with the recent studies by Cowan et al showing that serine proteinase and MMP activation are critical for inducing the proliferative and migratory events that characterize pulmonary vascular remodeling secondary to monocrotaline administration. In these studies, regression of pulmonary artery hypertension was observed after treatment with a serine elastase inhibitor, involving a loss of both cellular and matrix components. In the present study, aggravation of hypoxic PH was induced by treatment with doxycycline or lung overexpression of hTIMP-1, which both inhibit MMPs but not serine elastase. The experimental models also differ because monocrotaline-induced PH is associated with a marked inflammatory component not observed during exposure to chronic hypoxia. It is therefore likely that differences in the experimental models used and in the proteolytic enzymes targeted by the inhibitors may explain the apparent contrasting results between these studies.

The absence of difference in the Fulton index between doxycycline-treated rats and controls contrasts with the observation that pulmonary artery pressure and muscularization were increased in the doxycycline-treated group. However, one hypothesis could be that inhibition of MMPs by the systemic route could influence heart growth. The heart/body weight and left ventricular weight + sum of septum/body weight ratios support this hypothesis, as they were significantly lower in the doxycycline-treated rats than in the control rats, whereas right ventricle/body weight ratio was not. We are currently investigating this hypothesis. From our point of view, demonstration of increased pulmonary artery pressure and muscularization after inhibition of MMPs is a strong argument for aggravation of PH.

In conclusion, our study provides strong support for the hypothesis that MMPs may play a crucial role in pulmonary artery remodeling and may partially protect against PH development, as MMP inhibition led to aggravated PH. In view of recent results on treatment of monocrotaline-induced PH by elastase inhibition, further studies are clearly needed to evaluate the effect of MMP inhibition in such a model to develop new therapeutic strategies.

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References


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Figure 1 online: Gelatin zymography on lung homogenates.

Total gelatinase activity after SDS PAGE separation of MMPs from inhibitors in the lungs of rats exposed to chronic hypoxia and treated with either Ad.hTIMP-1 or Ad.Null (panel a) or doxycycline (panel b) were similar. 72 kD and 92 kD bands represent gelatinase A and B respectively.

Panel a: a-d: Ad.Null, e-h: Ad.hTIMP-1

Panel b: a-f: Control, g-l: Doxycycline