Differential response of TIMP-3 null mice to the lung insults of sepsis, mechanical ventilation, and hyperoxia

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Martin, Erica L., Lynda A. McCaig, Brent Z. Moyer, M. Cynthia Pape, Kevin J. Leco, James F. Lewis, and Ruud A. W. Veldhuizen. Differential response of TIMP-3 null mice to the lung insults of sepsis, mechanical ventilation, and hyperoxia. Am J Physiol Lung Cell Mol Physiol 289: L244–L251, 2005. First published April 1, 2005; doi:10.1152/ajplung.00070.2005.—An imbalance in matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) leads to excessive or insufficient tissue breakdown, which is associated with many disease processes. The TIMP-3 null mouse is a model of MMP/TIMP imbalance, which develops air space enlargement and decreased lung function. These mice responded differently to cecal ligation and perforation (CLP)-induced septic lung injury than wild-type controls. The current study addresses whether the TIMP-3 knockout lung is susceptible to different types of insults or only those involving sepsis, by examining its response to lipopolysaccharide (LPS)-induced sepsis, mechanical ventilation (MV), and hyperoxia. TIMP-3 null noninjured controls of each insult consistently demonstrated significantly higher compliance vs. wild-type mice. Null mice treated with LPS had a further significantly increased compliance compared with untreated controls. Conversely, MV and hyperoxia did not alter compliance in the null lung. MMP abundance and activity increased in response to LPS but were generally unaltered following MV or hyperoxia, correlating with compliance alterations. All three insults produced inflammatory cytokines; however, the response of the null vs. wild-type lung was dependent on the type of insult. Overall, this study demonstrated that 1) LPS-induced sepsis produced a similar response in null mice to CLP-induced sepsis, 2) the null lung responded differently to various insults, and 3) the null susceptibility to compliance changes correlated with increased MMPs. In conclusion, this study provides insight into the role of TIMP-3 in the lungs of sepsis, mechanical ventilation, and hyperoxia.

MATERIALS AND METHODS

Animal breeding. A full description of the generation and breeding of the TIMP-3 knockout mouse was previously reported (23, 28). All mice were housed and provided free access to standard rodent chow and water in an automatically controlled facility providing a 12:12-h light-dark cycle. All animals used were 9–12 wk of age, and all procedures were approved by the Animal Use Sub-Committee of the University of Western Ontario.

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**Experiment 1: LPS administration.** TIMP-3 wild-type and knockout animals were randomized to receive a 0.1 ml intraperitoneal (ip) injection of either 30 μg of LPS (from *Escherichia coli* 0111:B4; Sigma, Ontario, Canada) or saline control, resulting in four experimental groups: 1) (+/++) saline, 2) (+/++) LPS, 3) (−/−) saline, and 4) (−/−) LPS. Six hours after injection, animals were killed and weighed, and lungs were excised and analyzed as described in *Lung analysis*.

**Experiment 2: isolated-perfused mechanical ventilation.** TIMP-3 wild-type and knockout animals were anesthetized with an injection of 260 mg/kg pentobarbital sodium ip (MTC Pharmaceuticals, Cambridge, Canada) and randomized to nonventilation or ventilation, resulting in four experimental groups 1) (+/++) nonventilated, 2) (+/++) ventilated, 3) (−/−) nonventilated, and 4) (−/−) ventilated. Ventilated mice were prepared and perfused in situ as described previously by von Bethmann et al. (46). In brief, a catheter was inserted into the pulmonary artery, through which the lung vasculature was perfused in a noncirculating system. Throughout ventilation, 5-ml aliquots of perfusate, composed of RPMI 1640 lacking phenol red + 2% low endotoxin grade bovine serum albumin (Sigma), were collected following lung perfusion through a second catheter inserted in the left atria. Aliquots were snap frozen in liquid nitrogen for subsequent cytokine analysis. Lungs were ventilated by volume-cycled, positive pressure ventilation using the RV5 Rodent ventilator (Volkert Enterprises, Toronto, Canada) at 40 breaths/min, tidal volume of 10 ml/kg and zero peak end expiratory pressure. After ventilation, lungs were excised and analyzed as described in *Lung analysis*.

**Experiment 3: hypoxia exposure.** TIMP-3 wild-type and knockout animals were randomized to 48 h of 21% oxygen or >90% oxygen exposure (2), resulting in four experimental groups 1) (+/++) normoxia, 2) (+/++) hypoxia, 3) (−/−) normoxia, and 4) (−/−) hypoxia. A sealed Plexiglas box (50 x 50 x 32.5 cm; Parker Plastic, London, ON, Canada) was filled with the appropriate gas mixture using a gas mixer (model 3500HL; Sechrist Medical Products Division, Anaheim, CA) and the hospital’s medical air and oxygen lines, after which the mice were placed inside for duration of 48 h. Boxes were kept in a 12:12-h light-dark-cycled room that was temperature controlled, and mice were allowed free access to food and water. The oxygen levels inside the box were verified every 12 h (2). After 48 h, the animals were killed and weighed, and lungs were excised and analyzed as described in *Lung analysis*.

**Lung analysis.** For each experiment, excised lungs were analyzed for compliance via static pressure-volume curves to a maximum pressure of 25 cmH2O, using a Harvard Apparatus syringe pump (Harvard Apparatus Canada, Saint-Laurent, QC, Canada) and pressure monitor as previously described (45). All compliance measurements were completed by an investigator blinded to mouse genotype. After compliance measurements, the lungs were lavaged three times with 2 x 1 ml 0.15 M NaCl. Lung tissue was snap frozen in liquid nitrogen and stored at −70°C. The lavage was centrifuged at 200 g for 10 min at 4°C, and the supernatant was frozen in liquid nitrogen and stored at −70°C for cytokine analyses. The concentrations of IL-6, TNF-α, and transforming growth factor (TGF)-β were measured with separate OPTI-EIA ELISA kits specific for each cytokine following the manufacturer’s protocols (PharMingen, San Diego, CA).

Zymography was used to analyze MMP levels as previously described (28). In brief, lungs were homogenized in extraction buffer containing 50 mM Tris-Cl (pH 7.5), 5 mM CaCl2, 5 μM ZnCl2. Gels were stained in Coomassie brilliant blue dye for 60 min (Bio-Rad Laboratories, Hercules, CA) and destained for 15 min. Zymography was analyzed by spot densitometry using an Alpha Innotech Imager 2200 and AlphaEase image analysis software (Alpha Innotech, San Leandro, CA). Clear resolution of the gelatin zymography allowed for individual quantification of latent and active forms of MMP-2 and MMP-9. However, lower resolution of casein zymography (a limitation of this analysis) prevented accurate quantitative separation of the latent and active bands, resulting in combined analysis of MMP-8 and MMP-7. Identical gels that were incubated in incubation buffer containing 5 mM EDTA resulted in a complete lack of bands, confirming that the observed proteolytic activity was due to MMP activity.

**Statistical analysis.** All values are expressed as means ± SE. We determined significance by a two-way ANOVA when comparing four experimental groups and by a t-test when comparing two groups. Statistical analysis was performed with the SPSS statistical software package for Windows, version 9.0 (SPSS Chicago, IL). Differences were considered statistically significant when the probability value was <0.05. All significant results are displayed where a = (−/−) vs. (+/+), b = insult vs. control, and c = combined effect of (−/−) and insult.

**RESULTS**

Animals used in all studies were male mice between 9 and 12 wk of age, with weights and lavage volumes that were not significantly different among all groups.

**LPS administration.** After LPS, wild-type and knockout mice had decreased alertness and responsiveness, increased piloerection, abnormal posture or gait, and loose feces. These visual cues of a septic response occurred in both the wild-type and knockout mice and were observed by an investigator blinded to the type of injection and mouse genotype.

Compliance was measured by pressure-volume analysis (Fig. 1). Six hours following LPS injection, wild-type mice had lung compliance similar to saline-injected mice. TIMP-3 knockout mice injected with saline had significantly higher compliance compared with wild-type controls. Additionally, TIMP-3 knockout mice had significantly increased compliance following LPS administration compared with knockout saline animals.

MMP levels as detected by gelatin and casein zymography were analyzed by spot densitometry and were expressed relative to the wild-type saline animals, as displayed in Fig. 2. After LPS administration, wild-type mice had increased MMP-2 (active form), MMP-9 (active and latent forms), MMP-8, and MMP-7 compared with saline-injected wild-type controls. TIMP-3 null saline-injected mice had comparable MMP levels to wild-type saline controls. Similar to wild-type mice, null animals responded to LPS with increased MMP-2 (active form), MMP-9 (active and latent forms), MMP-8, and MMP-7 compared with saline-injected null controls. MMP-7 showed the largest response to LPS, as seen in Fig. 2D, with approximately a sixfold increase in relative intensity compared with saline controls.

Concentrations of IL-6, TNF-α, and TGF-β were measured in lavage fluid (Table 1). After LPS, wild-type mice had a significant increase in IL-6 levels compared with saline-injected controls. TIMP-3 knockout saline-treated mice had IL-6 levels that were not significantly different from those of wild-type controls. Knockout mice responded similarly to wild-type controls with an increase in IL-6 levels following LPS admin-
administration. TNF-α levels were below detectable levels in all experimental groups. TGF-β was not significantly different following LPS administration in the wild-type or knockout animals.

Isolated-perfused ventilation. Pressure-volume analysis of lung compliance (Fig. 3) showed that 90 min of ventilation produced a small, nonsignificant decrease in lung compliance in wild-type animals. TIMP-3 knockout mice had significantly higher lung compliance compared with wild-type controls, and the compliance of ventilated knockout mice was not significantly different from nonventilated knockout mice.

MMP-2, MMP-9, MMP-8, and MMP-7 levels are displayed in Fig. 4. There was a significant increase in MMP-2 (active form) and a significant decrease in MMP-2 and MMP-9 (latent form) in mechanically ventilated wild-type and null mice.

<table>
<thead>
<tr>
<th>Cytokine Levels</th>
<th>(+/+ Saline)</th>
<th>(+/+ LPS)</th>
<th>(−/− Saline)</th>
<th>(−/− LPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6, pg/ml</td>
<td>6.5±1.4 (6)</td>
<td>81.9±21.8 (6)*</td>
<td>4.3±1.1 (8)</td>
<td>61.7±6.2 (7)*</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>ND (6)</td>
<td>ND (6)</td>
<td>ND (8)</td>
<td>ND (7)</td>
</tr>
<tr>
<td>TGF-β, pg/ml</td>
<td>147±11 (6)</td>
<td>111±17 (6)</td>
<td>238±30 (8)</td>
<td>133±5 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Statistical significance at P < 0.05: b = LPS vs. saline. *P < 0.05, wild-type; −/−, knockout; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor; ND, not detectable. Numbers in parentheses indicate number of animals examined (n).

Table 1. Cytokine levels in whole lung lavage as measured by ELISA following LPS administration in TIMP-3 wild-type and knockout mice

Fig. 2. Matrix metalloproteinase (MMP) levels as analyzed by gelatin and casein zymography, for the 4 experimental groups as described in Fig. 1. Values are presented as relative values compared with the wild-type, saline group. Open bars represent the latent form of the MMP, whereas solid bars represent the active form. A: MMP-2 levels (72 kDa), B: MMP-9 levels (105 kDa), C: MMP-8 levels (75 kDa) (latent and active forms combined in analysis), D: MMP-7 levels (28 kDa) (latent and active forms combined in analysis); n = 4 in all groups. Statistical significance at P < 0.05: b = LPS vs. saline.
compared with nonventilated controls. MMP-8 levels were not significantly different among experimental groups, whereas TIMP-3 null mice responded differently to ventilation than wild-type mice through a decrease in MMP-7.

Table 2. Cytokine levels in whole lung lavage as measured by ELISA following isolated-perfused mechanical ventilation in TIMP-3 wild-type and knockout mice

<table>
<thead>
<tr>
<th></th>
<th>(+/+) Nonvent</th>
<th>(+/-) Vent</th>
<th>(-/-) Nonvent</th>
<th>(-/-) Vent</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 levels, pg/ml</td>
<td>40±8 (7)</td>
<td>198±18 (6)</td>
<td>45±10 (6)</td>
<td>112±10 (6)</td>
</tr>
<tr>
<td>TNF-α levels, pg/ml</td>
<td>ND (6)</td>
<td>48±10 (5)</td>
<td>9±10 (6)</td>
<td>5±10 (7)</td>
</tr>
<tr>
<td>TGF-β levels, pg/ml</td>
<td>47±26 (7)</td>
<td>52±22 (6)</td>
<td>27±18 (5)</td>
<td>63±22 (7)</td>
</tr>
</tbody>
</table>

Statistical significance at P < 0.05: b = ventilated (Vent) vs. nonventilated (Nonvent); c = combined effect of (−/−) and ventilation.

Analysis of IL-6, TNF-α, and TGF-β cytokines in the lavage are shown in Table 2. IL-6 levels were significantly increased in wild-type mice following ventilation compared with nonventilated controls. Knockout nonventilated mice were not significantly different from wild-type nonventilated mice, and knockouts also showed a significant increase in IL-6 following ventilation vs. knockout nonventilated animals. However, the concentration of IL-6 following ventilation was significantly less in knockout animals compared with wild-type controls. In lavage, TNF-α levels were not significantly different among the experimental groups; however, there was a trend of increased TNF-α levels in wild-type lungs compared with knockout lungs following ventilation [P = 0.077 for the combined effect of (−/−) and ventilation]. TGF-β levels were not significantly different between the experimental groups in lavage.
Figure 5 displays cytokine levels in perfusate throughout ventilation. Similar to lavage, IL-6 and TNF-α concentrations in wild-type perfusate increased over the course of ventilation. Although TIMP-3 knockout mice also demonstrated increased IL-6 levels in perfusate, by 90 min of ventilation the concentration was significantly lower than wild-type controls. Following the same trend as IL-6, there was no significant change in TNF-α levels following ventilation of the TIMP-3 knockout animals. TGF-β in the perfusate was not significantly different between wild-type and knockout animals until 90 min of ventilation, at which time TIMP-3 knockout animals released significantly more TGF-β than wild-type controls.

Hyperoxia exposure. Figure 6 shows that following 48 h of hyperoxic exposure there was a significant increase in compliance in wild-type mice. There was significantly higher compliance in the knockout normoxic mice compared with wild-type normoxic mice. However, following hyperoxic exposure the TIMP-3 knockout mice did not show a significant increase in compliance.

MMP-2, MMP-9, MMP-8, and MMP-7 levels are displayed in Fig. 7. After hyperoxic exposure, wild-type mice showed no alterations in MMPs compared with normoxic controls. TIMP-3 null mice had increased MMP-2 (active form) compared with wild-type normoxic controls; however, all other MMP levels were not significantly different. Similar to wild-type, TIMP-3 null mice exposed to hyperoxia did not have any alterations in MMP levels.

Cytokine concentrations for IL-6, TNF-α, and TGF-β are shown in Table 3. IL-6 and TNF-α were increased in wild-type mice following hyperoxic exposure compared with normoxic controls. Cytokine levels in TIMP-3 knockout normoxic mice were not significantly different from wild-type controls. Wild-type lungs responded to hyperoxia with a significant increase in IL-6 and TNF-α compared with normoxic wild-type controls. However, both IL-6 and TNF-α concentrations in the knockout hyperoxic lungs were significantly larger than wild-type hyperoxic lungs. TGF-β was significantly increased in wild-type mice in response to hyperoxia compared with normoxia. TGF-β from TIMP-3 knockout normoxic mice was not significantly different from wild-type controls. Null mice also responded to hyperoxia with a significant increase in TGF-β.
However, the increase in TGF-β in the knockout hyperoxic mice was significantly lower than that of the wild-type hyperoxic mice.

**DISCUSSION**

This study addressed the question of how TIMP-3-deficient mice, which have an endogenous MMP/TIMP imbalance, respond to different lung insults. The results demonstrated that the responses varied greatly based on the type of insult. Compared with the wild-type mice, TIMP-3 knockout animals had increased lung compliance and increased MMP activity after receiving the septic insult, but not after a mechanical ventilation or hyperoxic insult. Additionally, the inflammatory cytokine response varied between the three insults tested. These results led to the conclusion that TIMP-3 does not have one specific role in the lung but, rather, is involved in a variety of processes within the lung including maintenance of normal lung compliance, as well as both up- and downregulation of the inflammatory response. It was also observed that in all three models of lung stress, the measured MMP levels were, for the most part, similar between wild-type and TIMP-3 knockout animals. This leads us to conclude that although TIMP-3 is known to regulate MMP activity, it is not directly involved in the regulation of MMP protein levels in response to insults. It should be noted that, similar to most studies using knockout animals, a limitation of this study is that we are unable to distinguish whether the response to insults in the TIMP-3 knockout lung is a result of the absence of TIMP-3 directly or whether it is the result of the altered lung phenotype. Further studies are required to address this issue.

Lung compliance was analyzed as an indication of lung function. After LPS administration, lungs from TIMP-3 null mice developed a significant increase in lung compliance compared with saline-treated null controls. These results mirror those found in our previous study using the CLP model of sepsis, which indicated that the increased compliance is likely due to degradation of collagen and fibronectin in the lung (28). Although a decrease in compliance is conventionally thought to be indicative of poor lung function (2, 45), an increase in compliance is also reflective of poor function as seen in diseases such as emphysema (26). Conversely to LPS, both mechanical ventilation and hyperoxic exposure insults did not induce increased lung compliance in the TIMP-3 knockout mouse. Therefore we can conclude that this unique and rapid alteration in lung compliance in TIMP-3 null mice is a response specific to an indirect inflammatory septic insult. Although wild-type mice did not have altered compliance follow-

**Table 3. Cytokine levels in whole lung lavage as measured by ELISA following 48 h of either normoxic or hyperoxic exposure in TIMP-3 wild-type and knockout mice**

<table>
<thead>
<tr>
<th></th>
<th>(+/+ Norm)</th>
<th>(+/+ Hyp)</th>
<th>(-/- Norm)</th>
<th>(-/- Hyp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 levels, pg/ml</td>
<td>31±8 (5)b</td>
<td>42±2 (5)a</td>
<td>50±6 (5)abc</td>
<td></td>
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<tr>
<td>TNF-α levels, pg/ml</td>
<td>16±2 (5)a</td>
<td>ND (4)a</td>
<td>44±3 (5)abc</td>
<td></td>
</tr>
<tr>
<td>TGF-β levels pg/ml</td>
<td>181±49 (8)</td>
<td>133±33 (5)b</td>
<td>324±48 (5)abc</td>
<td></td>
</tr>
</tbody>
</table>

Norm, normoxia; Hyp, hyperoxia. Statistical significance at P < 0.05: a = (-/-) vs. (+/+), b = hyperoxia vs. normoxia, c = combined effect of (-/-) and hyperoxia.
ing either LPS or mechanical ventilation, wild-type mice exposed to hyperoxia developed increased lung compliance, which is consistent with some previous reports (10, 20), although other studies have reported a decrease or no change in compliance (2, 44).

Because TIMP-3 is an endogenous inhibitor of MMPs (37) and excessive tissue breakdown by MMPs is known to increase lung compliance (28, 34), we speculate that increased degradation by MMPs causes the altered compliance following a septic insult in the TIMP-3 knockout animals. Although TIMP-3 wild-type and null mice have similar amounts of MMPs, the TIMP-3 null mice have less inhibition of their MMPs due to the lack of TIMP-3 in vivo. Therefore, the MMPs present in the null lung exert a greater degree of damage. Our previous paper supports this concept since TIMP-3 null mice had similar MMP levels to wild-type controls but had higher MMP activity as assessed by in situ zymography (28). The current study further supports this concept since LPS, which increased compliance, was shown to increase several MMPs, specifically MMP-7. Furthermore, mechanical ventilation and hyperoxia insults, which did not induce compliance alterations in the null mouse, produced very few or no increases of the analyzed MMPs. Although these experiments do not show a cause-and-effect relationship, they provide strong evidence that TIMP-3 knockout mice are susceptible to sepsis due to increased MMP activity. Additionally, since MMP-7 is known to degrade collagen and fibronectin (21), both of which are reduced in the TIMP-3 null mouse following sepsis (28), and is most predominantly increased following the septic insult, we speculate that MMP-7 is a major cause of the increased compliance in the TIMP-3 null mouse in response to a septic insult. A corollary of this is that TIMP-3 plays an important role in the regulation MMP-7 activity.

Recent publications have demonstrated that TIMP-3 may be involved in the regulation of inflammation (6, 30). In an analysis of our results of inflammatory cytokines, a specific role for TIMP-3 is difficult to define. The cytokines we chose to analyze are IL-6, TNF-α, and TGF-β, since IL-6 and TNF-α are commonly used as markers of inflammation (11, 25, 42), and TGF-β levels have been linked with altered MMP levels (7). All three insults were vigorous enough to produce an inflammatory IL-6 signal in the wild-type lung, indicating a proinflammatory lung insult. The lack of TNF-α at 6 h following the LPS insult may simply reflect the time course TNF-α secretion since this cytokine is known to peak early in the inflammatory cascade, ~1 h following insult (17). After LPS administration, TIMP-3 knockout mice showed a similar increase in cytokine levels as wild-type controls. However, following mechanical ventilation, TIMP-3 null mice produced less cytokines than wild-type controls, and conversely, TIMP-3 null mice exposed to hyperoxia produced more cytokines than their wild-type controls. Overall, these results provide evidence that the role of TIMP-3 in the regulation of inflammation may be dependent on the specific mechanisms through which the inflammatory insult is produced. For example, TIMPs are known to have several alternative functions such as altering various signaling pathways, including the MAP kinase pathway and PKA pathway, or inhibition of members of the ADAM family, both of which may influence cytokine expression (22, 29).

Whereas the above discussion focuses on the comparisons among the three different insults, it is also worthwhile to discuss the implications of our study within some of the individual models. For example, several studies have demonstrated that during ventilation-induced lung injury MMPs are increased in the lung (14, 16). In contrast, our experiment found significant decreases in MMPs following ventilation that may be related to the specific model or ventilation strategy utilized. Interestingly, we observed that TIMP-3 knockout mice had significantly lower inflammatory cytokine concentrations in lung lavage and perfusate following ventilation compared with wild-type controls. Therefore, it is possible that TIMP-3 null mice may be protected from the inflammatory aspects of ventilation-induced lung injury.

Conversely to ventilation, TIMP-3 mice produced a larger inflammatory response following hyperoxia vs. wild-type controls. This is of particular interest since the air space enlargement phenotype of the TIMP-3 mouse resembles that of pulmonary emphysema (23), and a major clinical therapy of emphysema is oxygen therapy (39). Our studies suggest that clinical use of hyperoxic therapy may induce higher levels of inflammation in an environment of MMP/TIMP imbalance, which is known to exist in lung diseases such as emphysema, pulmonary fibrosis, and acute lung injury (33).

In summary, TIMP-3 had been identified as essential for 1) maintenance of a normal lung phenotype by regulating tissue turnover in the lung and 2) protection of the lung in response to a CLP-induced septic insult (23, 28). The current experiments build on this original information by determining that the role of TIMP-3 in the response to injury is dependent on the type of insult. We have confirmed that TIMP-3 null mice are specifically susceptible to sepsis, induced by either CLP or LPS administration, and that this susceptibility is most likely due to upregulated MMPs. Additionally, we suggest that specifically MMP-7 plays a pivotal role in the response to sepsis.

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GRANTS

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