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“Deficiency of Matrix Metalloproteinase-13 Increases Inflammation after Acute Lung Injury”

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Abstract

Human and animal studies of acute lung injury (ALI) have shown that matrix metalloproteinases (MMPs) play an important role in disease pathogenesis, but despite being detected during ALI, the function of the collagenase MMP-13 in ALI is unknown. To evaluate this role of MMP-13, mice deficient in MMP-13 (KO) were examined after hyperoxic lung injury, and compared to wild-type (WT) mice. There was no survival difference between KO and WT mice. There was also no difference in fibrosis between WT and KO mice, as determined by hydroxyproline content and collagen expression by real-time PCR. Within the BAL, the KO mice exhibited a significant increase in inflammatory cells, when compared to the WT mice (5.51 vs. 2.35×10^5 cells/mL; $p = .001$). Increased levels of the chemokine MCP-1 were observed in the lungs of the KO mice, confirmed via immunohistochemistry. In a subsequent *in vitro* experiment, MMP-13 was shown to cleave MCP-1. In ALI in the MMP-13 KO mice, MCP-1 could therefore remain active and potentially attract macrophages to the BAL. This study suggests a direct role for MMP-13 in modifying the inflammatory response in the lung after ALI.

Keywords

MCP-1; hyperoxia; MMP-13; acute lung injury; knockout mice

Introduction

Acute lung injury (ALI) is a complex pathophysiologic process with great importance to the clinical patient population. After initial lung injury, whether by pneumonia, sepsis or trauma, the body responds with varying degrees of fibrosis, fluid leak, and inflammation. The degree of this response and the subsequent repair can have a significant effect on long term outcome after injury (1). Understanding the molecules that control this response may lead to new and innovative ways to treat ALI.

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Declaration of Interest

The authors report no conflicts of interest and are alone responsible for the content and writing of this paper.

Matrix metalloproteinases (MMPs) are enzymes important for extracellular matrix remodeling and degradation, and have a complex relationship with various disease processes, including atherosclerosis, hepatic fibrosis, and lung injury (15). Typically secreted as inactive molecules, MMPs are tightly regulated at several levels, including transcription and activation (14), and target a variety of substrates, from extracellular membrane components to membrane receptors and chemokines (6, 20, 27). Previous studies suggest a role for MMPs in the pathophysiology of lung injury, and suggest that MMPs could prove valuable targets in the clinical treatment of ALI (4, 5, 8, 10, 13, 24, 25, 28).

The focus of the present study, murine MMP-13, the major collagenase in mice, cleaves multiple extracellular matrix substrates as well as several cytokines (15, 20). In humans, a mutation in MMP-13 causes a skeletal disorder known as the Missouri variant of spondyloepimetaphyseal dysplasia, and MMP-13 knockout mice (KO) show similar defects in bone remodeling (22). In addition, MMP-13 KO mice develop reduced hepatic fibrosis after cholestatic injury (26). The involvement of MMP-13 in acute lung injury, by contrast, has not been well-studied, despite MMP-13 being detected post injury. Since MMP-13 has previously been shown to affect both fibrosis and regulators of inflammation, our goal was to describe the role of MMP-13 in acute lung injury. We exposed MMP-13 deficient mice to hyperoxia and then compared the response to that in wild type controls, with the purpose of identifying the role of MMP-13 in the pathophysiology of acute lung injury.

Materials and Methods

Matrix Metalloproteinase-13 Deficient Mice

MMP-13 KO mice were created as previously described (9, 23). Four month old mice were used in this study, divided evenly between males and females. MMP-13 KO mice were compared with their WT littermates. They were bred and maintained on a C57BL6 background.

Acute Lung Injury Model

All procedures involving animals were performed following review by the Institutional Animal Care and Use Committee (Columbia University, New York, NY). The model for hyperoxic acute lung injury was based on standard models published previously (2, 11, 12, 16, 17). During exposure to hyperoxia, mice in their cages were placed in an airtight, plastic box. The mice were allowed food and water *ad libitum*. Pressurized liquid oxygen was mixed with medical air to create the atmosphere of 85% oxygen. The hyperoxic conditions were confirmed daily with a percent oxygen sensor (Nuvair Pro O₂ Remote Analyzer). The humidity and temperature were measured on a daily basis, and maintained within normal range (30-70% humidity, and 68-72°F). Based on the volume of the box, the gas flow rate was calculated to give at least twelve volume changes per hour. Additionally, soda lime was placed outside the cage but within the chamber to facilitate carbon dioxide absorption. Mice were maintained on a 12-hour light-dark cycle, and the chambers were opened once daily for change of water, bedding, and food. Mice were tested under two general conditions. One group of mice was exposed to 85% oxygen for three (n=6), five (n=6) or six (n=6) days. A second group of mice was exposed to 85% oxygen for three (n=6) or six (n=28) days, followed by seven days of recovery in room air, with water and food *ad libitum*. After this exposure to hyperoxia or hyperoxia with rest, the mice were sacrificed, and lung, BAL, and serum samples were collected.

Tissue Preparation

Following mice being sacrificed, the trachea was blunt dissected and cannulated with a 20 French plastic cannula. Sterile PBS was used to perform the bronchoalveolar lavage (BAL).

BAL fluid was collected, the right lung flash frozen in liquid nitrogen for the harvest of protein and RNA, and the left lung perfused with formalin for histology. A tail snippet was stored at -20°C for confirmatory mouse genotyping. Lung tissue slides were subsequently stained with Hematoxylin and Eosin, Masson's Trichrome, and Picrosirius Red, using standard procedures.

Morphometric Analysis of Lung Tissue Sections

After Hematoxylin and Eosin staining of lung sections, fifteen 40x images of the lung from each mouse were obtained to calculate the fractional volume of parenchymal tissue per lung, the alveolar surface area per unit volume, and the mean linear intercept as per standard protocol (7). Briefly, sixteen parallel horizontal lines, each 6.7 mm in length, were overlaid on each image. The number of times a line intercepted an alveolar wall was counted as the line count, and the number of times a line ended on an alveolar wall was counted as the par count.

FITC-Albumin Analysis

To assess the protein leak in the acute phase of ALI, mice were exposed to 3, 5, and 6 days of 85% oxygen. Mice were tail vein injected with 200 µl of a 0.75 mg/ml FITC-Albumin solution (Sigma Product #A9771) 2 hours before they were sacrificed. Immediately upon sacrifice and blunt dissection, the right ventricle of the heart was cannulated with a 23 gauge needle, and approximately 1 ml of blood was evacuated. This blood was allowed to clot on the benchtop at room temperature and then centrifuged at -4°C at maximal speed for 15 minutes. The serum was then collected. A total of 2 ml of sterile PBS was infused into the trachea in 1 ml aliquots, which was spun at -4°C at 3000 rpm for 10 minutes. The supernatant was collected, and the cell pellet was set aside. The spun BAL and serum (50 µl each) were placed into separate fluorometer cuvettes, and the fluorescence of each was measured. The ratio of BAL fluorescence to serum fluorescence was calculated, with higher ratios representing more fluid leak and thus lung damage.

Hydroxyproline Content

Equal masses of lung tissue for KO and WT mice were lyophilized overnight, and then reconstituted in 4 µl of 6N HCl. These samples were then placed on a heating block at 116 °C for 16 hours. One µl of this hydrolysate was evaporated, and reconstituted with one µl distilled water. These samples were re-evaporated, and reconstituted with 5 µl distilled water. Two µl of each sample solutions were mixed with one µl Chloramine-T for 20 minutes at room temperature. One µl of 3.15 M perchloric acid was added and the sample incubated for 5 minutes at room temperature. Next, one µl of p-DMABA was added, and the solutions mixed. The solutions were incubated at 60°C for 20 minutes, cooled for 5 minutes, and absorbance readings taken at 557 nm. The sample solutions were compared to a prepared hydroxyproline standard.

Real Time Polymerase Chain Reaction (RT-PCR)

Real Time PCR analysis was used to evaluate the mRNA levels of MMP-13, the fibrillar collagens (collagen I and collagen III), and elastin. RNA was harvested from lung tissue using the RNeasy Mini Kit (Qiagen), transformed into a cDNA library, and used for the TaqMan RT-PCR assay using manufacturer's protocols.

Bronchoalveolar Lavage

After tracheal instillation of 2 mL (divided into two 1 mL aliquots) of sterile PBS, the BAL was obtained and centrifuged at 4°C for 10 minutes at 3000 rpm. The cell pellet was

resuspended in 300 µl of sterile PBS and a cell count was performed using a hemocytometer. The cell pellet was spun via cytospin onto a microscope slide for histology.

TUNEL Analysis

Lung sections were tested for apoptosis using the Roche *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science) using the manufacturer's protocol. Quantification of apoptotic cells was performed by counting the number of TUNEL-positive cells in 15 images (20x magnification) from each mouse tested. The percentage of apoptotic cells was obtained by dividing this count by the total number of nuclei (stained with DAPI) counted per slide.

Cytokine Array

Lung tissue protein samples were tested for levels of 62 pre-determined cytokines via a cytokine array (RayBio Catalog # AAM-CYT-3-4). Lung tissue samples from MMP-13 WT and KO mice that were exposed to 6 days of 85% oxygen followed by 7 days of rest in room air were incubated as per the manufacturer's recommendations, and screened for 62 cytokines. The protein arrays were then electronically scanned, and densitometry was performed on the dots of the WT and KO mice (Carestream Molecular Imaging Software). The mean intensities of the WT and KO dots were compared after subtracting out the mean intensity of the positive controls.

Immunohistochemistry

Sections from the formalin-fixed and paraffin embedded samples were deparaffinized and microwave-treated for 10 minutes in pH 9.0 Tris-HCl buffer for antigen retrieval. After immersion in 3% H₂O₂ to block endogenous peroxidase activity, the slides were incubated with goat polyclonal antibody against mouse MCP-1 (1:100; R&D Systems, Inc., product #AF479-NA) in 5% BSA/PBS at 4°C overnight. Incubation with non-immune goat immunoglobulin (IgG) (1 µg/ml, Abcam Inc.) in 5% BSA/PBS at 4°C overnight was used as a control. After reaction with antibody against biotin-conjugated goat IgG (1:2000; Jackson ImmunoResearch Laboratories, Inc.) and peroxidase-labeled Avidin-Biotin Complex (Vector Laboratories), color was developed with DAB. The sections were then counterstained with hematoxylin and observed using a light microscope.

In vitro digestion of MCP-1 by MMP-13

Recombinant human MMP-13 (R&D Catalog #511-MM) and carrier-free recombinant murine MCP-1 (R&D Catalog #479-JE/CF) were tested *in vitro* to determine the ability of MMP-13 to cleave MCP-1. Recombinant MMP-13 was activated by incubation with APMA at 37°C for 1 hour. MMP-13 was combined with 50 ng of MCP-1 for 8 hours at 37°C, at varying molar ratios from 0:1 to 30:1. The samples were then run on a 17% SDS-PAGE gel at 100 volts for 2.5 hours and subsequently silver stained (Waco Silver Staining Kit.)

Statistical Analysis

Using the Student's two-tailed *t*-test, significance was determined by a threshold of 0.05, with variability represented by standard error of the mean in the figures and tables presented. The sample size was a minimum of 6 animals per exposure group, up to 28 animals per exposure group.

Results

No Difference in Mortality of MMP-13 WT and KO Mice after Hyperoxic Lung Injury

Mortality rates for the KO and WT mice after exposure to hyperoxia were similar for each condition tested. After exposure to 6 days of 85% oxygen followed by 7 days of rest on room air (6d7R), the mortality rates were 21.4% (3/14) for WT mice and 14.3% (2/14) for KO mice. The WT and KO mice had a mortality rate of 0% (0/11) after 3 days of 85% oxygen, a mortality rate of 0% (0/6) after 5 days of 85% oxygen, and a mortality rate of 0% (0/6) after 3 days of 85% oxygen followed by 7 days of rest on room air. The mortality rate after 6 days of 85% oxygen was not calculated as the mice were ill-appearing and sacrificed.

No Difference in Protein Leak of MMP-13 Mouse Lungs after Hyperoxic Lung Injury

To determine the degree of acute protein leak seen after hyperoxia, mice were tested using the FITC-Albumin assay. There was no significant difference between WT and KO mice after three days (0.096 vs. 0.112, $p=NS$), five days (0.089 vs. 0.102, $p=NS$) or six days (0.075 vs 0.097, $p=NS$) of 85% oxygen with no rest in room air, as demonstrated by the ratio of fluorescence in the BAL to serum. (Figure 1)

No Difference in Fibrosis in MMP-13 Mouse Lungs after Hyperoxic Lung Injury

There was no significant disparity in fibrosis between the MMP-13 KO and WT mice after lung injury. Histologically, both Masson Trichrome and Picrosirius Red staining were similar between the WT and KO 6d7R samples. (Figure 2A) The hydroxyproline content between WT and KO mice was not significantly different when tested after three days of 85% oxygen with seven days of rest (0.129 vs. 0.136), or after six days of 85% oxygen with seven days of rest (0.133 vs. 0.132). (Figure 2B and Table 1) Similarly, RT-PCR analysis exhibited no significant difference in the level of collagen I, collagen III, or elastin when WT and KO mice were compared (data not shown). There was no significant difference in lung morphometry as determined by surface area per unit volume, fractional volume, or mean linear intercept when comparing the WT and MMP-13 KO mice both after room air only and after exposure to six days of 85% oxygen followed by seven days of rest (Table 2).

Upregulation of MMP-13 mRNA Expression in WT Mice after Hyperoxic Exposure

After exposure to 6 days of 85% oxygen followed by 7 days of rest (6d7R), MMP-13 mRNA expression in MMP-13 WT mice was upregulated 5.2-fold when compared to MMP-13 mRNA expression of MMP-13 WT mice exposed only to room air. As expected, there was no MMP-13 mRNA expression in the 6d7R MMP-13 KO mice.

Increased Inflammatory Cell Infiltration in the BAL of MMP-13 KO Mice

In the 6d7R groups, there was significantly more inflammation in the BAL of the KO mice when compared to the WT mice. The average cell count in the BAL of the KO mice was 5.51×10^5 cells/mL, compared to the WT mice with an average cell count of 2.35×10^5 cells/mL ($p=.001$). (Figure 3 and Table 1) Macrophages were the predominant cell type (99%) in both the KO and WT groups, with a small number of neutrophils (1%). (Figure 4) Interestingly, the lung tissue from the 6d7R KO mice did not exhibit more inflammatory cells histologically, when compared to control littermates (data not shown). Also of note, the degree of inflammation in the BAL after 6 days of hyperoxia with 7 days of rest was increased over baseline room air BAL cell count in both the KO mice (0.70×10^5 cells/mL) and WT mice (0.39×10^5 cells/mL). No significant difference in BAL or lung inflammation existed between WT and KO mice when they were exposed to only 3 days of 85% oxygen with 7 days of rest.

Mild Increase in Apoptosis seen in MMP-13 KO Mice after Hyperoxic Injury as Determined by TUNEL Assay

A low level of apoptosis was detected by TUNEL assay in both the WT and MMP-13 KO 6d7R mice. The degree of apoptosis after hyperoxic exposure (0.039% WT vs. 0.066% KO) was slightly increased overall from room air baseline (0.008% WT vs 0.012% KO). (Table 1) Also, the hyperoxia-exposed MMP-13 KO mice showed slightly more apoptosis than did the hyperoxia-exposed WT mice, with a p value < .05.

Increased Cytokine Levels in the Lungs of MMP-13 KO Mice

To further characterize the mechanism behind the increased inflammation, a protein array was performed using homogenized lung tissue from WT and KO MMP-13 6d7R mice. The array of 62 proteins demonstrated increased levels of the cytokine MCP-1 in the lung tissue of the KO mice when compared with the WT mice, with a ratio of 1.52 to 1. To validate this finding, formalin-fixed slides of lung tissue from 6d7R mice were stained for MCP-1 using immunohistochemistry. Increased MCP-1 staining was seen in the lungs of the MMP-13 KO mice, predominantly within the macrophages and alveolar epithelium. (Figure 5) Out of the remaining 61 cytokines in the protein array, only CD-40 and VCAM-1 showed a difference in expression (CD-40 was 1.11-fold increased in the lungs of the WT mice, and VCAM-1 was 2.94-fold increased in the lungs of the WT mice), but when validation studies were performed at the protein level, no difference in the levels of these two molecules was observed between the WT and KO mice.

MMP-13 Cleaves MCP-1 in vitro

As further validation of the hypothesis that MMP-13 inactivates MCP-1, an *in vitro* experiment combining activated MMP-13 and whole MCP-1 demonstrated that at molar concentrations of 20:1 and 30:1, MMP-13 cleaves MCP-1 into two fragments. (Figure 6) In the WT mice, the cleavage of MCP-1 by MMP-13 may inactivate the cytokine, explaining why in the ALI model, MMP-13 KO mice with intact MCP-1 have more inflammation in the BAL after hyperoxic lung injury.

Discussion

MMP-13 is the primary interstitial collagenase in rodents, important in bone remodeling and hepatic injury, yet its role in lung injury has yet to be defined. MMP-13 expression was found in this investigation to be upregulated more than 5-fold in the WT mice after hyperoxic insult, suggesting an active role for MMP-13 in ALI. Under the condition of MMP-13 deficiency, this study examined three main indicators of acute lung injury: protein leak, fibrosis, and inflammation. Although no significant differences were discovered in terms of protein leak or fibrosis, MMP-13 KO mice had significantly more inflammation in their BAL than their WT counterparts after exposure to hyperoxia followed by rest in room air.

The MMP-13 KO mice exhibited more inflammatory cells in their BAL, but did not demonstrate increased inflammation in lung tissue under histological examination. Using immunohistochemistry, we demonstrated higher levels of MCP-1 in the lungs of MMP-13 KO mice when compared to WT mice after hyperoxic injury. We then demonstrated that MMP-13 cleaves MCP-1 *in vitro*. Our hypothesis is that MMP-13 cleaves and inactivates MCP-1, a cytokine important for regulating inflammation after acute lung injury, and in MMP-13 KO mice, MCP-1 remains active with a subsequent increase in inflammation. Even though MCP-1 is likely one of multiple cytokines potentially regulated by MMP-13, these findings begin to describe a novel role for MMP-13 in ALI in an *in vivo* model. The ability for MMPs to modulate other proteins has been described in several *in vitro* systems

(20, 21), but recently, there has been more evidence showing that MMPs are active in live models (19). The work that we present here adds to that growing body of evidence, expanding the role of MMPs in live models, and bringing us closer to understanding the biology of MMPs in injury and repair.

Similarly, the role of gelatinolytic MMPs in regulating inflammation has been described in lung injury models other than ALI. For example, in allergic lung disease models (3, 18), both MMP-2 and MMP-9 were shown to influence inflammation after injury, both in the BAL and within the lung parenchyma. Also, studies that investigate the enzymatic properties of MMPs (20), have confirmed that MMPs not only act on elements of the extracellular matrix but are also capable of cleaving and potentially inactivating cytokines. With regard to the cleavage of MCP-1 by MMP-13, our data is in conflict with that of McQuibban et al (20). Technically, there are several differences in the methods employed. In particular, the molar ratio used in the two studies was different, with the McQuibban study using 100-fold more substrate, which could explain the different results. However, the body of evidence detailed above combined with the data presented in this paper expands the role of MMPs beyond that of matrix remodeling and suggests that MMPs may also have a direct role in affecting cell movement and inflammation.

Despite the significant difference in inflammation seen after ALI, there was no difference in mortality or morbidity noted between the MMP-13 WT and KO mice, and no difference in the pathology seen within the damaged lung tissue, including both basic histology and lung morphometry. A significant increase in macrophages was seen in the BAL, but we were surprised that increased inflammation was not observed within the lung tissue itself. To investigate this inflammatory effect further, we performed a TUNEL assay to assess the degree of apoptosis seen after hyperoxic injury. There was a slight increase in apoptosis noted in the MMP-13 knockout mice after hyperoxic injury (0.039% WT vs 0.066% KO). In this model, there was no observable histological difference between the wild type and MMP-13 knockout mice, despite the slightly increased ratio of apoptosis. Therefore, the potential effect of MMP-13 on apoptosis may better be assessed using alternative injury models. Overall, these findings suggest that although inflammation is an important component of ALI, its contribution to pathological changes after injury in ALI needs to be defined more clearly. Other regulators of inflammation within the lung tissue itself may play a greater role in determining outcome after ALI, and should be investigated further.

Given that MMP-13 has been shown previously to regulate fibrosis in a hepatic model (26), it was somewhat surprising that deficiency of MMP-13 prompted no difference in fibrosis after ALI when compared to WT mice. There are a number of possible explanations for this finding. The role of MMP-13 may not be the same in hepatic injury as compared to lung injury, either because of the model used or the pathophysiology of the disease. Another possibility is that in the ALI model system, there is an insignificant development of fibrosis before sacrifice. We hypothesized that one week of rest on room air was a reasonable amount of time to develop fibrosis given that the mice recovered clinically within a few days of being in room air, but perhaps using a later time point would help distinguish differing degrees of fibrosis. Also, although fibrosis develops in the human lung after ALI, the mouse model may repair itself more efficiently with less resultant fibrosis. The hyperoxia model is ideal for examining the acute effects of lung injury, which may explain why although there was an increase in BAL macrophages in the MMP-13 KO mice, the lung pathology, in terms of fibrosis, in the WT and KO mice was similar.

In this paper, we show one aspect of the response to injury after acute lung injury, but we acknowledge that the physiologic picture is much more complicated. Other proteins, perhaps even alternate MMPs, may regulate other cytokines besides MCP-1 after injury, in

conjunction with MMP-13's effect on MCP-1. Although it is very difficult to parse out the role of a single protein in an intricate pathophysiology such as ALI, knockout models can help isolate the role of one protein. Ultimately, one's findings need to be put in a greater perspective, and continued research into the many functions of MMPs is needed. With this investigation, the role of MMP-13 can be redefined to include the regulation of inflammation after ALI.

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Figure 1. Alveolar Protein Content as Determined by FITC-Albumin Ratio. No significant difference is seen in the acute alveolar protein leak between MMP-13 KO and WT mice, as measured by FITC-Albumin ratio (BAL:serum). Mice were tested after exposure to oxygen: 3, 5, and 6 days (n=6 per exposure group) of 85% oxygen with no rest in room air. (Results represented by mean value + SEM)

Figure 2. Fibrosis in the Injured Lung. **A:** Masson Trichrome and Picrosirius Red Staining of Injured Lungs. No qualitative difference in the degree of fibrosis between MMP-13 WT and KO mice after ALI with 6 days of 85% oxygen and 7 days in room air rest. Original magnification, $\times 40$. **B:** Hydroxyproline Content in the Lungs. No significant difference in hydroxyproline content is seen within the lung tissue between MMP-13 KO and WT mice. Mice were tested after exposure to oxygen: 3 days of 85% oxygen with 7 days of rest in room air ($n=6$), and 6 days of 85% oxygen with 7 days of rest in room air ($n=6$). (Results represented by mean value + SEM)

Figure 3.

Bronchoalveolar Lavage Cell Count. Increased inflammation is seen in the BAL of MMP-13 KO mice (5.51×10^5 cells/mL) when compared to WT mice (2.35×10^5 cells/mL) after 6 days of 85% oxygen followed by rest for 7 days in room air ($n=28$, * $p = .001$, Student's t -test). No significant increase is seen in MMP-13 WT and KO mice in room air ($n=8$) or exposed to 3 days of 85% oxygen followed by 7 days of rest in room air ($n=6$). (Results represented by mean value + SEM)

Figure 4. Macrophages in Bronchoalveolar Lavage Cytospin. Significantly more macrophages seen in the BAL of MMP-13 KO mice after 6 days of 85% oxygen followed by 7 days of rest in room air. **A:** BAL from WT mice after 6 days of 85% oxygen and 7 days of rest in room air, showing predominantly macrophages. **B:** BAL from KO mice after 6 days of 85% oxygen and 7 days of rest in room air, showing predominantly macrophages. **C:** Magnified image from WT mouse BAL. **D:** Magnified image from KO mouse BAL. (A and C, ×40; B and D, ×100).

Figure 5.

MCP-1 Immunohistochemistry for lung tissue shows significantly more staining for MCP-1 in MMP-13 KO mice when compared to WT mice. **A:** WT lung after 6 days of 85% oxygen, followed by rest for 7 days in room air. **B:** KO lung after 6 days of 85% oxygen, followed by rest for 7 days in room air. (**A** and **B**, $\times 40$).

Figure 6.

In vitro MMP-13 digestion of MCP-1. At molar concentrations of MMP-13: MCP-1 of 20:1 and 30:1, MMP-13 clearly cleaves MCP-1 into a smaller fragment, as demonstrated on this silver stained gel.

Table 1

Lung Injury in MMP-13 Wild Type and Knockout Mice.

	<u>Hydroxyproline Assay</u>	<u>BAL Cell Count</u>	<u>TUNEL Assay</u>
	<u>Absorbance (A557)</u>	<u>Cell Count per mL $\times 10^5$</u>	<u>Apoptotic Nuclei/Total Nuclei (%)</u>
Room Air Only (n=8)			
WT	n.d.	0.39 $\pm$ 0.12	0.008 $\pm$.001
MMP-13 KO	n.d.	0.70 $\pm$ 0.13	0.012 $\pm$.004
3d 85% Oxygen, 7d Rest Room Air (n=6)			
WT	0.129 $\pm$ 0.002	1.12 $\pm$ 0.13	n.d.
MMP-13 KO	0.136 $\pm$ 0.002	1.26 $\pm$ 0.35	n.d.
6d 85% Oxygen, 7d Rest Room Air (n=6: Hydroxyproline and TUNEL) (n=28: BAL Cell Count)			
WT	0.133 $\pm$ 0.001	2.35 $\pm$ 0.36	0.039 $\pm$.004
MMP-13 KO	0.132 $\pm$ 0.003	5.51 $\pm$ 0.73 [*]	0.066 $\pm$.002 [*]

Mean Values $\pm$ SEM; n.d. = not determined^{*}
p < 0.05

Table 2

Morphometry Measurements of MMP-13 WT and KO Mice.

	<u>Surface Area/Unit Volume</u>	<u>Fractional Volume %</u>	<u>Mean Linear Intercept</u>
<u>Room Air Only</u> (n=6)			
WT	111.6 ± 4.5	35.8 ± 1.8	17.6 ± 0.7
MMP-13 KO	110.1 ± 0.6	33.8 ± 0.1	18.0 ± 0.0
<u>6d 85% Oxygen, 7d Rest Room Air</u> (n=6)			
WT	101.4 ± 1.0	26.3 ± 1.3	19.6 ± 0.3
MMP-13 KO	92.3 ± 5.1	29.6 ± 2.3	21.6 ± 1.2

Fractional volume of parenchyma tissue per lung {V(p/l)}; Alveolar surface area per unit volume {S(p/l)(mm⁻¹); mean linear intercept {microns}; Mean Values ± SEM