

Localization of Matrix Metalloproteinase (MMP)-9 in Lung Tissue of a Murine Model of Allergic Asthma

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MMP-9 (gelatinase B) is recognized in chronic obstructive pulmonary disease (COPD) and now asthma as playing a central role in matrix degradation in injury, as well as contributing to the remodeling process. The increasing focus on MMP-9 in human and animal research supports the need for a reliable immunostain in lung tissue. However, MMP-9 immunostaining in murine systems is hampered by several factors. First, many of the anti-human antibodies do not readily cross-react with murine MMP-9 despite the high degree of conservation between human and murine MMP-9. Secondly, the availability of detailed protocols is limited. Lung MMP-9 immunostaining is further complicated by technical issues such as edge effect, availability of positive and negative controls, antigen retrieval, staining specificity, and the need to achieve a delicate balance of primary and secondary antibody concentrations, and colorimetric reagents which will allow visualization of specific cell expression in highly delicate lung tissue, while also demonstrating adequate uptake in (extra-pulmonary) tissue controls. We describe a detailed method for immunostaining MMP-9 in mouse lung paraffin-**embedded** tissue utilizing human ovary as a control since MMP-9 is known to be over-expressed in human ovarian carcinomas.

Keywords Matrix Metalloproteinase-9 (MMP-9), Asthma, Immunostain.

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INTRODUCTION

The field of matrix biology has expanded rapidly over the past decade. Lung research has increasingly been directed toward clarifying the key components in lung inflammation and remodeling, in COPD, asthma and lung injury. Central to this remodeling is the role of matrix metalloproteinases (MMPs), their expression, and regulation. Asthma is a chronic inflammatory disease of the airways wherein MMPs, bound to cells or elaborated into the extracellular matrix, are induced and cause lung pathophysiology (Crosby & Waters 2010; Todorova L et al 2010; Baines et al 2009). Long-term management of asthma requires control of inflammation and prevention of secondary lung remodeling (Cataldo et al., 2002; Hegele, 2000). Asthma exacerbations can increase MMP-9 production, upsetting its normal balance with the expression of its specific inhibitor, TIMP-1, resulting in damage to the extracellular matrix of the lung followed by abnormal repair or remodeling.

MMPs are important in inflammatory cell migration, healing and pathologic remodeling in many tissues, including the lung (Atkinson et al., 2011; Atkinson and Senior, 2003; Cataldo et al., 2002; Ohbayashi, 2002; Ohbayashi & Shimokata, 2005). A paradox in asthmatic lung pathophysiology is the recognition that inflammation and remodeling may be concurrent rather than sequential events. MMP-2, -9 and -12, which are central to collagen turnover, are upregulated in human and animal models of asthma suggesting that they are pivotal in the injury-healing and pathologic remodeling cycle. Understanding what up-regulates pathologic lung remodeling in asthma instead of normal healing is fundamental to the development of the next generation of asthma therapy. MMP-9 is active against gelatin, collagens IV and V, elastin, and proteoglycan core protein; its activities are inhibited by TIMP-1 (Woessner, 1998). MMP-9 is required for airway epithelial migration in matrix remodeling. Inhibition of MMP-9 activity in a murine model of allergic asthma induced by chicken ovalbumin (OVA) attenuated the cytokine, cellular and tracheal inflammatory responses (Kumagai et al., 1999; Leong and Huston, 2001; Pauwels et al., 1997; Vignola et al., 2000;).

Limited reports on MMP-9 immunostaining in murine systems are available and additionally many of the MMP-9 anti-human antibodies do not cross-react with murine MMP-9 despite the high degree of conservation between human and murine MMP-9. Therefore we developed and standardized a detailed method for immunostaining MMP-9 in mouse lung paraffin-embedded tissue (Betsuyaku et al., 2000; Yaguchi et al., 1998; Lemjabbar et al., 1999; Fukuda et al., 1998; Hayashi et al., 1996). The objectives of this investigation were 1) development of an effective MMP-9 immunostain in murine lung tissue and 2) evaluation of MMP-9 localization in lung tissue of a murine model of allergic asthma induced by chicken OVA.

METHODS

Study Design

All experiments performed were approved by the IACUCs of the University of Buffalo and also the Veterans Administration. We recently validated, and described a murine model of allergic asthma consisting of a tissue inhibitor of metalloproteinase (TIMP)-1 genetic knockout (KO) on a C57Bl/6 background (Sands et al., 2009). An established, homozygous breeding colony of a TIMP-1 genetic knockout (KO) mice (backcrossed >10 generations from 129/sv) and their wild type C57Bl/6 backcross controls was initially provided by Dr. Paul Soloway of Cornell University.

These KO mice were maintained in a pathogen-free laminar flow holding unit and the food was autoclaved. We and others demonstrated that the C57Bl/6 strain develops immune and reactive airway responses to OVA sensitization (Sands et al., 2009). This model allows us to examine a permissive, disinhibited environment for MMP-9 activation. C57Bl/6 mouse littermates were randomly assigned to be sensitized with Sigma Grade III OVA-alum by intra-peritoneal injection on days 1 and 14, followed by inhalation of nebulized OVA on days 21-27 inclusive. Following euthanasia, lungs were harvested on day 29 after inflation with buffered formalin. Lungs were fixed for 72 hr, then paraffin embedded and sectioned, and mounted on glass slides (Figure 1) (Sands et al., 2009). Human ovarian carcinoma tissue was obtained in accordance with university and hospital ethical guidelines, to serve as a tissue control. A total of 85 animals were analyzed for IgE studies and the histopathology studies included 20 animals per cohort, totaling 80 animals.

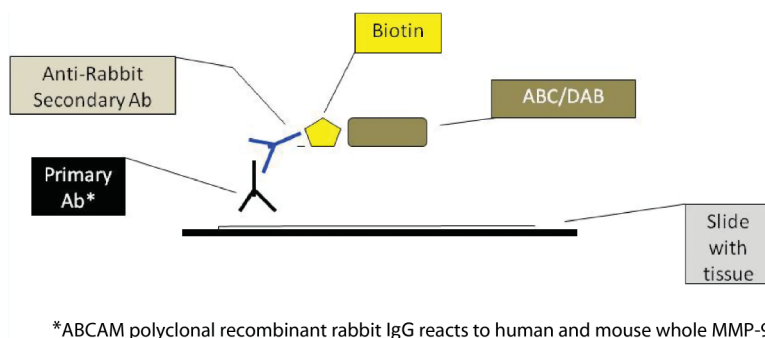


Figure 1: Schematic of the MMP-9 immunostaining protocol.

Genotype Confirmation of C57BL/6 Homozygous TIMP-1 Genetic KO Mice

We confirmed the genotypic background of the TIMP-1 genetic KO mice using PCR amplification of DNA extracted from tails of the C57BL/6 homozygous TIMP-1 genetic KO mice. Our data showed differential migration patterns of C57BL/6 TIMP-1 KO and WT animals demonstrating that the TIMP-1 gene disruption is stable and does not suffer reversion to the wild type (WT) (Sands et al., 2009).

Acute sensitization protocol. On day 0, C57BL/6 mice ages 6–8 wk, are injected intra-peritoneally (i.p.) with 10 μ g OVA (Grade III, Sigma, St. Louis MO) + 1 mg of the adjuvant, AlKSO₄(H₂O)₁₂, or saline (SHAM group) in 0.2 ml of sterile phosphate-buffered saline (PBS). This is followed by daily 30 min aerosol exposures of 1% OVA (wt/vol) or saline (SHAM group) on days 14–21 delivered by an ultrasonic nebulizer (Figure 2) (DeVilbiss Ultraneb 99, Somerset, PA) (Korsgren et al., 1997; Tormanen et al., 2005).

ELISA for total murine IgE and OVA-specific IgE to confirm allergic sensitization. Mouse total IgE is measured in pooled sera from 15 littermates of identical OVA-immunized or control mice by a commercial assay using purified mouse IgE standards as described by the manufacturer (PharMingen, San Diego, CA). OVA-specific IgE is a variation of the total IgE assay using a double-sandwich, biotinylated ELISA according to the manufacturer's protocol from PharMingen. The ELISA shows a sensitive, linear dose-response relationship between optical density and titer of OVA-specific IgE in mouse serum and no cross-reaction with unrelated IgE or IgG. Each mouse group acts as its own negative control by comparing pre-sensitization to post-sensitization titers. Our results (Figure 3) show that our sensitization protocol induced significant levels of OVA-specific IgE in C57BL/6 mice.

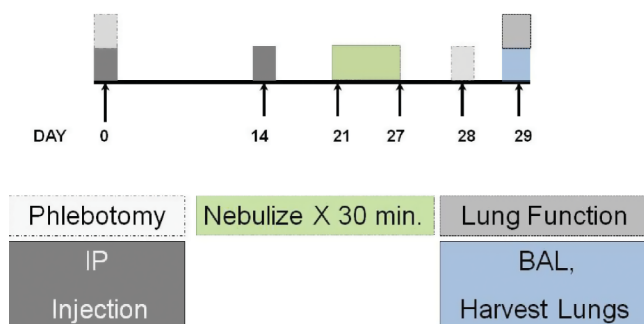


Figure 2: Schematic of the acute sensitization protocol.

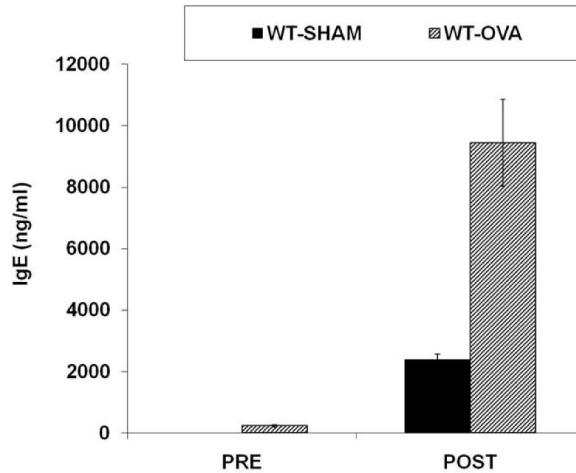


Figure 3: ELISAs from plasma for OVA-specific IgE showing undetectable levels in un-sensitized mice on day 1 ("Pre") as compared to readily detectable, reproducible amounts in the same mice following sensitization on Day 28 ("Post"), and between sham and OVA-sensitized POST groups. $P = 0.0001$ (ANOVA).

Histopathology

The lungs are fixed by inflating to a pressure of 20 cm H₂O with vapor-phase 16% formaldehyde (37°C) while suspending the lungs in 10% neutral buffered formalin (37°C) for 18 hr. Gross cross-sections (2–3 mm) of each lobe were embedded in paraffin blocks, and fine sections (4 μm) were cut with a microtome and stained with hematoxylin and eosin (H&E), collagen (trichrome), and periodic acid-Schiff (PAS) for light microscopy. Lung orientation for cutting and paraffin embedding was adapted from Ellis et al. (2003) with modification to optimize visualization of relatively uniform sizes of cross cut bronchi for quantitative analysis (Sands et al., 2009).

A pathologist, blinded to the experimental group, determined inflammation scores for 10 bronchi (100–150 μm diameter) per mouse for 20 mice in each experimental group. Inflammation was graded 0 to V as described by Tournoy et al. (2000) with modifications as follows: Grade 0 = no inflammation; Grade I = occasional bronchial cuffing with inflammatory cells; Grade II = >50% of bronchi surrounded by a thin layer (1–4) of mononuclear cells or eosinophils; Grade III = >50% of bronchi surrounded by a thick layer (5–8) of mononuclear cells or eosinophils; Grade IV = >50% of bronchi surrounded by a layer of >9 cells; Grade V = Grade IV with prominent lymphoid aggregates and interstitial infiltration. Differential and total cell counts are determined for SHAM and OVA treated groups using H&E stain. At least 20 high power fields per experimental group are sampled, generating a differential cell count of $\geq 1,000$ cells (OVA-treated groups), and ≥ 200 cells (SHAM groups).

MMP-9 Immunostaining

- Deparaffinization and rehydration. Slides are immersed in 100% xylene 3 times for 5 min. They are then dehydrated by immersion in ethanol (EtOH) for 3 min at concentrations of 100%, 90%, 70%, 50% and then distilled water (d-H₂O).
- Endogenous Peroxidase Blockade. Slides are immersed in 3% H₂O₂ for 10 min.
- Epitope (antigen) retrieval. Citrate buffer solution is prepared by combining 4.1 ml sodium citrate to 0.9 ml of 0.1M citric acid, bringing the total volume to 50 ml by adding d-H₂O. Slides are placed in a Coplin jar containing 40ml of this citrate buffer solution. The Coplin jars are placed in a microwave oven and heated at 700 Watts for 2 min, then 70 Watts for 10 min and allowed to cool for 15 min in the buffer solution. Placing 2 additional buffer-filled Coplin jars (without slides) in the oven is advised to even the energy transfer to the slides. Slides are then rinsed by immersion for 5 min in PBS_T (0.1% Tween-phosphate buffered solution).
- Protein block. Casein (Sigma #C5890) 0.3% solution (in PBS_T) is prepared in advance, and stirred 8–12 hr (overnight). Slides are immersed in blocking solution for 30 min.
- Tissue is encircled on the slide with a pap-pen (Zymed, San Francisco).
- Primary antibody. Rabbit Polyclonal IgG whole-molecule (anti-MMP-9 mouse/human; Abcam Inc., Cambridge, MA #16306-50) is prepared from previously frozen aliquots. It is diluted to 1 mcg/ml in PBS.
- Isotype control (for primary antibody). ChromPure Rabbit IgG, whole molecule from Jackson ImmunoResearch Laboratories, West Grove, PA (#011-000-003) is utilized as there is no peptide blocking antibody available for this primary antibody. It must be diluted to the same concentration as the primary antibody, in this case 1 mcg/ml. It is applied each run to identical lung sections as a negative control. The primary and isotype control antibodies are incubated for 60 min at room temperature (RT), then rinsed 3 times for 3 minutes in PBS_T.
- A PBS control slide with no primary (or isotype control) antibody is also run as above.
- Biotinylated secondary antibody. Biotinylated Anti-rabbit IgG (H + L) (#BA-1000) (Vector Laboratories, Burlingame, CA) is utilized at a starting concentration of 1.5 mcg/ml to prepare a 1:250 dilution in water. Incubate 60 min at RT. Rinse 3 times for 3 min with PBS_T by immersion with gentle agitation to remove excess antibody.

- Avidin plus biotinylated horse radish peroxidase (ABC). Prepare 30 min before use, diluting according to the manufacturers instructions (Santa Cruz). Incubate for 30 min at RT. Rinse twice for 3 min in PBS_T.
- Chromogen. Liquid DAB Substrate Chromogen System (Dako Inc ,Carpenteria, CA) is applied for exactly 2 min. It should be gently and thoroughly rinsed by immersion or running d-H₂O over the slide for 5 min.
- Counterstain. Hematoxylin (Vector Laboratories, Burlingame, CA) (H3404) is applied to the slide for 30 sec, then rinsed with H₂O for 5 min. Filtration is not needed for fresh hematoxylin.
- Dehydrate and overlay with a coverslip. In distinct jars from the re-hydration step, immerse slides, for 3 min intervals, in 70%, 90%, and then twice in 100% EtOH. Next, immerse in 100% xylene 3 times in 3 separate containers, for 3 min each.
- Overlay immediately with a coverslip.

RESULTS AND DISCUSSION

In the current investigation we utilized an acute sensitization protocol (Figure 2) in our TIMP-1 KO murine model of allergic asthma. In this model we have shown that baseline physiology is altered compared to WT animals, causing aberrations in lung development (Sands et al., 2009). The ELISAs for total IgE and OVA-specific IgE confirm allergic sensitization of the animals and our results show that our sensitization protocol induces significant levels of OVA-specific IgE in C57Bl/6 mice (Figure 3). Additionally, our results show that OVA-sensitized murine lung tissue demonstrates eosinophilic, monocytic and lymphocytic infiltration and significant levels of MMP-9 expression (Figure 4).

The immunohistochemical localization of MMP-9 using the above staining methodology showed that there was a significant increase in MMP-9 expression in the lungs of the TIMP-1-KO animals compared to wild-type, both in the OVA sensitized and the sham groups. Biotinylated anti-mouse MMP-9 (IgG) results in uptake in multiple cell types of murine lung tissue. This protocol results in enhanced zymographic MMP-9 activity in the lungs of sensitized mice versus sham-sensitized lung as reported previously (Sands et al., 2009).

No inflammation was observed in either SHAM sensitized group. Mild to moderate inflammation (Grades II-IV) was observed in the WT-OVA group whereas sensitization in the TIMP-1 KO-OVA group was associated with predominantly moderate to severe inflammation (Grades III-V) (Sands et al., 2009). The inflammation in both sensitized groups consisted predominantly of lymphocytic and eosinophilic peribronchial and perivascular infiltrates (Sands et al., 2009).

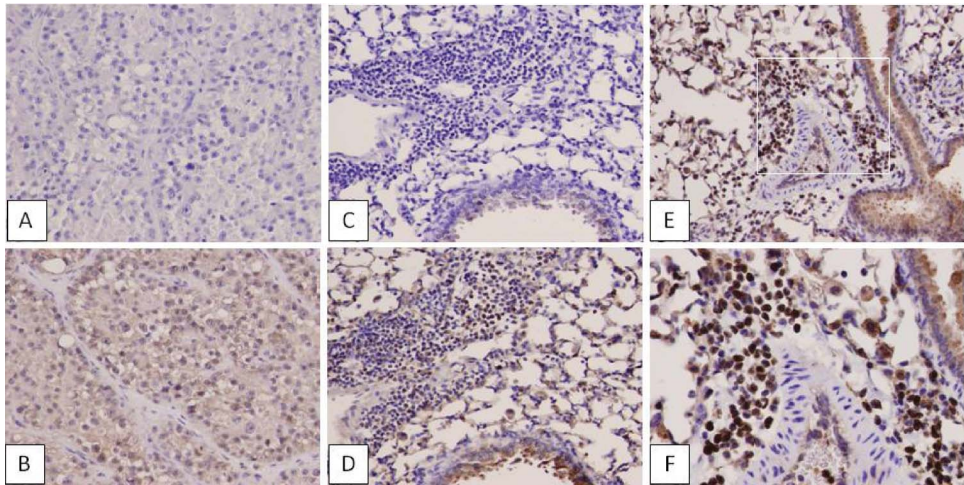


Figure 4: A. Human ovarian carcinoma MMP-9 negative control; B. Human ovarian carcinoma showing positive MMP-9 immunostaining; C. Lung tissue from OVA-sensitized TIMP-1 KO mouse negative control; D. Serial section to C depicting positive MMP-9 immunostaining; E. Low power of lung tissue from OVA-sensitized TIMP-1 KO mouse showing extensive MMP-9 immunostaining of peri-bronchial cellular infiltrates and epithelial surfaces of airway; F. High power view of a selective area of E showing details of positive MMP-9 immunostaining of epithelial cells, eosinophils, and mononuclear and lymphocytic infiltrating cells.

Immunostaining (CD86+) for monocyte/macrophages revealed significant increases (compared to SHAM) in sensitized WT and TIMP-1 KO mice, but we observed no genotypic effects. Infiltrates were most intense at areas deep to bronchial bifurcations. Trichrome staining for collagen revealed deposition of collagen within the inflammatory infiltrates (Sands et al., 2009). All histopathological analyses were performed by a pathologist blinded to the study groups.

MMP-9 is known to be expressed in epithelial cells as well as neutrophils, monocyte/macrophage cells, and lymphocytes. This staining technique demonstrates uptake in each of these cells, demonstrating that the biotinylated MMP-9 immunohistochemical method described allows precise localization of MMP-9 in lung tissue for use in murine lung research. An additional advantage of this immunohistochemical protocol is that background staining is minimized with this methodology. Our goal was to optimize the MMP-9 immunostaining method in our model system prior to subsequent experiments examining inhibition of MMP-9 gene expression by treatment of the animals with MMP-9 specific siRNA to reduce inflammation after OVA sensitization and challenge.

We hypothesize that administration of MMP-9 specific siRNA to the lungs of sensitized animals would abrogate development of an asthma phenotype, with endpoints of reduced airway hypersensitivity, less inflammation and

tissue remodeling, less cellular migration of lymphocytes and eosinophils, and less activation of inflammatory cells, in both BAL fluids and lung tissues. Additionally lung tissue remodeling, as manifested by collagen deposition in the sub-epithelial and basement membrane regions could be reduced in and consistent with reduction of MMP-9 expression. These experiments await completion.

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

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