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# A rapid increase in macrophage-derived versican and hyaluronan in infectious lung disease

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ABSTRACT

The goals of this study were to characterize the changes in chondroitin sulfate proteoglycans and hyaluronan in lungs in acute response to gram-negative bacterial infection and to identify cellular components responsible for these changes. Mice were treated with intratracheal (IT) live *Escherichia coli*, *E. coli* lipopolysaccharide (LPS), or PBS. Both *E. coli* and LPS caused rapid selective increases in mRNA expression of versican and hyaluronan synthase (Has) isoforms 1 and 2 associated with increased immunohistochemical and histochemical staining for versican and hyaluronan in the lungs. Versican was associated with a subset of alveolar macrophages. To examine whether macrophages contribute to versican and hyaluronan accumulation, *in vitro* studies with primary cultures of bone marrow-derived and alveolar macrophages were performed. Unstimulated macrophages expressed very low levels of versican and hyaluronan synthase mRNA, with no detectible versican protein or hyaluronan product. Stimulation with LPS caused rapid increases in versican mRNA and protein, a rapid increase in Has1 mRNA, and concomitant inhibition of hyaluronidases 1 and 2, the major hyaluronan degrading enzymes. Hyaluronan could be detected following chloroquine pre-treatment, indicating rapid turnover and degradation of hyaluronan by macrophages. In addition, the effects of LPS, the M1 macrophage classical activation agonist, were compared to those of IL-4/IL-13 or IL-10, the M2a and M2c alternative activation agonists, respectively. Versican and Has1 increased only in response to M1 activation. Finally, the up-regulation of versican and Has1 in the whole lungs of wild-type mice following IT LPS was completely abrogated in TLR-4$^{-/-}$ mice. These findings suggest that versican and hyaluronan synthesis may play an important role in the innate immune response to gram-negative lung infection.



## 1. Introduction

In the acute response to bacterial lung infection, gram-negative bacteria are recognized by toll-like receptors (TLRs) on the surface of alveolar macrophages triggering a cascade of events that leads to pulmonary inflammation and, ultimately, bacterial clearance and healing. TLR activation stimulates resident macrophages to secrete a variety of chemokines, cytokines and other molecules that induce recruitment of neutrophils and monocytes from the bloodstream. This leukocyte response is essential to the propagation and resolution of inflammation. We are interested in the potential for proteoglycans and related molecules secreted by macrophages to have a role in promoting the acute inflammatory response to bacterial infections in the lung.

Proteoglycans are important biological modifiers that influence both homeostasis and the response to injury (Esko and Lindahl, 2001; Parish, 2006; Gill et al., 2010). In the lung, proteoglycans also have a role in organ development and contribute to the innate immune response to infection (Gill et al., 2010; Tanino et al., 2012). Following the treatment of lungs with lipopolysaccharide (LPS), the glycosaminoglycan composition changes from predominantly heparan sulfate in healthy lungs to predominantly chondroitin and dermatan sulfate in inflamed lungs (Karlinsky, 1982; Blackwood et al., 1983). Changes in the composition of proteoglycans and their glycosaminoglycan chains, resulting in increases in versican, decorin and biglycan, are also documented in both animal models and human conditions of chronic lung disease (Karlinsky, 1982; Blackwood et al., 1983; Bensadoun et al., 1996, 1997; Malmstrom et al., 2002; de Medeiros Matsushita et al., 2005). Hyaluronan is a glycosaminoglycan that is not attached to a core protein, but binds to a number of chondroitin sulfate proteoglycans,

*Abbreviations:* TLRs, toll-like receptors; LPS, lipopolysaccharide; IT, intratracheal; PCR, polymerase chain reaction; IHC, immunohistochemistry; $C_t$, threshold cycle; b-HABP, biotinylated hyaluronan binding protein; BMDMs, bone marrow derived macrophages; Has, hyaluronan synthase; Hyal, hyaluronidase; ELSA, enzyme-linked sorbent assay; SEM, standard error of the means.

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including versican, to form large molecular weight complexes (Day and de la Motte, 2005). Similarly to versican, hyaluronan also is increased in chronic lung diseases (Hallgren et al., 1989; Nettelbladt and Hallgren, 1989; Nettelbladt et al., 1989; Jiang et al., 2005) and has been shown to have an important role in airway mucosal defense (Forteza et al., 2001).

Temporal and spatial changes in the expression of specific chondroitin sulfate proteoglycans and hyaluronan in the lungs during the innate immune response have not been systematically studied. Thus, the goals of this study were to characterize the changes in chondroitin sulfate proteoglycans and hyaluronan in lungs of mice with gram-negative bacteria and to identify specific cellular components that contribute to these changes. While multiple cell types are involved in the inflammatory response of the lungs (Strieter et al., 2002), this study focuses on the contribution of macrophages and epithelial cells as these are the cells that first encounter airborne pathogens and have been shown to have key roles in the initiation and regulation of inflammation in the lungs (Berg et al., 1993; Koay et al., 2002; Skerrett et al., 2004).

## 2. Results

### 2.1. Versican mRNA is Selectively Increased in Lungs of E. coli- and LPS-treated mice

To determine if acute inflammation alters the composition of chondroitin sulfate proteoglycans in the lungs, mice were exposed to IT live *E. coli* or PBS for 6 h and RNA was isolated from whole lung homogenates. Lungs from mice with *E. coli* pneumonia had a 33.2 ± 3.7-fold increase in versican mRNA, but no changes to the amounts of decorin or biglycan mRNA, as compared to mice treated with PBS (Fig. 1A). To identify changes in proteoglycan expression over time, subsequent studies used LPS to model gram-negative lung infection. LPS was used because this TLR-4 agonist provides a robust, more predictable and consistent activation of the innate immune system. Changes in the amount of mRNA for versican, decorin, and biglycan were measured using RNA isolated at 2, 6, and 24 h after treatment with LPS or PBS (Fig. 1B). There was significantly more versican mRNA in lungs of mice challenged with LPS vs. PBS at all three timepoints. LPS instillation caused a 4.4 ± 1.6-fold increase in versican at 2 h as compared to control lungs, a 33.2 ± 2.1-fold increase at 6 h, and a 4.45 ± 1.0-fold increase at 24 h. There were no changes in versican mRNA expression in the lungs of PBS-treated mice. There were also no changes in decorin or biglycan mRNA expression in the lungs of either LPS- or PBS-treated mice. These findings show that versican expression is rapidly and selectively upregulated in lungs in response to *E. coli* and LPS.

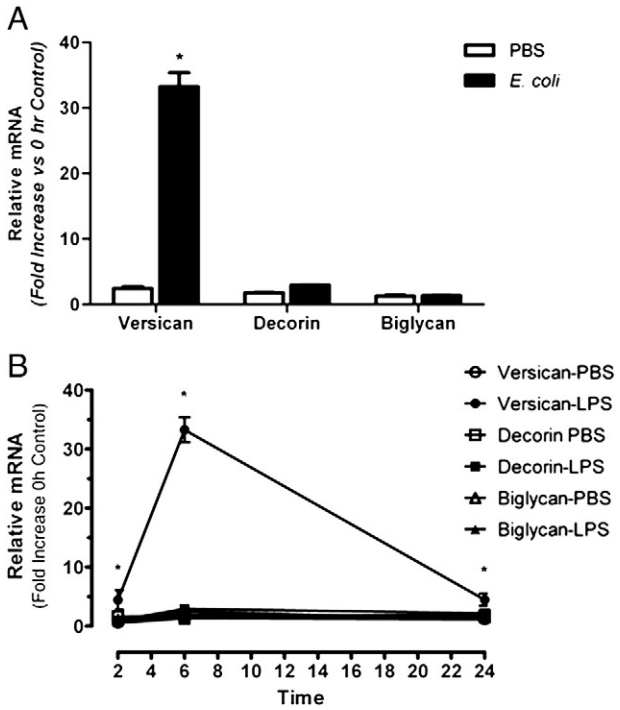


**Fig. 1.** Versican mRNA is increased in lungs of E. coli- and LPS-treated mice. Changes in the relative amounts of mRNA for the chondroitin sulfate proteoglycans, biglycan, decorin, and versican, were determined using mRNA collected from whole lung homogenates and quantitative real time PCR. A, Comparison of mRNA recovered from lungs of mice treated with PBS or 1 μg/g E. coli were made at 6 h. B, Comparison of mRNA recovered from lungs of mice treated with PBS or 1 μg/g LPS were made at 2, 6, and 24 h. Values are the mean ± SEM with a minimum n = 3 for each group studied. The expression of mRNA for each proteoglycan studied is expressed as a relative fold increase in mRNA over the 0 h control. An asterisk (*) shows groups that are significantly different ($p \leq 0.05$) when mice treated with PBS and LPS were compared.

### 2.2. Hyaluronan synthase mRNA is increased in lungs of E. coli- and LPS-treated mice

RNA collected from lungs of mice treated with live *E. coli* or LPS also was analyzed for changes in expression levels of the hyaluronan synthase isoforms. In mice treated with live *E. coli* for 6 h, there were significant increases in both Has1 (10.6 ± 1.3-fold increase) and Has2 (8.9 ± 1.0 fold-increase) mRNA, as compared to mice treated with PBS (Fig. 2A). In contrast, there was no change in the amount of Has3 mRNA (1.7 ± 0.6-fold increase). In mice treated with LPS, the expression of Has1 and Has2 mRNA was greatest at 2 h after IT instillation of LPS, but returned to basal levels at 24 h (Fig. 2B). When compared to control, LPS instillation caused 91.0 ± 12.8- and 10.5 ± 0.8-fold increases in Has1, and 21.6 ± 2.7- and 8.9 ± 0.6-fold increases in Has2 at 2 and 6 h, respectively. In contrast, Has3 mRNA was unchanged at 2 and 6 h after LPS instillation, but was significantly increased by 5.9 ± 1.0-fold at 24 h over the control (Fig. 2B). There were no changes in mRNA expression for any of the Has isoforms in PBS-treated mice. These findings show that Has1 and Has2 expression are rapidly and specifically increased, while Has3 expression is delayed in lungs in response to *E. coli* and LPS.

### 2.3. Versican and hyaluronan staining are increased in lungs of LPS-treated mice

To determine changes in their spatial localization in lungs, IHC for versican and affinity histochemistry for hyaluronan were performed on tissue samples obtained 6 h after the instillation of PBS or LPS. In the lungs of mice treated with PBS, a low level of positive staining for versican and hyaluronan was observed in the extracellular matrix of large airways and vessels (Table 1). The positive staining for versican and hyaluronan tended to be localized to the basal lamina of epithelial cells and in close proximity to the musculature of the vessels or airways. In these same mice, there was little to no staining for versican or hyaluronan in the lung parenchyma or pulmonary veins (Fig. 3). In mice treated with LPS, there was a clear increase in the positive staining for versican and hyaluronan around pulmonary veins (Fig. 3C and D), and an increase in the number of animals with positive staining for versican in the alveolar septa (Table 1). The majority of the positive staining for versican and hyaluronan appeared to be associated with the extracellular matrix, but intracellular staining of endothelial cells and/or lung fibroblasts could not be ruled out and needs to be investigated in future studies. Photomicrographs for the PBS- (Fig. 3A and B) and LPS-treated lungs (Fig. 3C and D) are representative images and show the same vessel in adjacent tissue sections. Photomicrographs of the PBS-treated lungs are of lower magnification to highlight the minimal amount of versican and hyaluronan in normal lungs. When lungs were evaluated for versican immunostaining, 4/6 (66%) of the mice treated with LPS had alveolar macrophages that stained positive and 3/6 (50%) had white blood cells that stained positive (Table 1 and