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Catalase attenuates pulmonary fibrosis while increasing pro-inflammatory cytokines

Lei Shi
University of Iowa

CATALASE ATTENUATES PULMONARY FIBROSIS WHILE INCREASING PRO-INFLAMMATORY CYTOKINES

by

Lei Shi

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Thesis Supervisor: Associate Professor A. Brent Carter

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Lei Shi

has been approved by the Examining Committee
for the thesis requirement for the Master of Science degree
in Human Toxicology at the May 2013 graduation.

Thesis Committee: _____
A. Brent Carter, Thesis Supervisor

_____
Douglas R. Spitz

_____
Larry W. Robertson

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ABSTRACT

Pulmonary fibrosis is an aberrant transformation of injured lung tissue. It is characterized by irreversible accumulation of extracellular matrix produced by fibroblasts and myofibroblasts during tissue remodeling, resulting in destruction and dysfunction of the lung. Asbestos is an important cause of pulmonary fibrosis. In response to asbestos exposure, alveolar macrophages and recruited monocytes generate reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), pro-inflammatory cytokines, such as TNF-α and IL-1β, and induce subsequent collagen deposition in the lung. We have found that increased H_2O_2 levels are linked to the development of pulmonary fibrosis. Catalase converts H_2O_2 into water and oxygen, so we hypothesized that catalase may attenuate the development of pulmonary fibrosis.

Interestingly, previous studies from our lab have demonstrated that decreased H_2O_2 levels are associated not only with a decrease in fibrosis but also an increase in pro-inflammatory cytokines. In these current studies, we demonstrate that in the presence of asbestos, catalase increases TNF-α and IL-1β in macrophages while decreasing collagen production in fibroblasts. This is reversed when TNF-α receptor-1 is knocked down, suggesting that TNF-α may play a role in fibrosis development. To investigate these finding *in vivo*, catalase-treated mice showed decreased fibrosis histologically, decreased in collagen levels in BAL, and decreased hydroxyproline in lung tissue.

The major finding of my study is that catalase attenuates asbestos-induced fibrosis while increasing pro-inflammatory cytokines. This is contrary to the typical thought that pro-inflammatory states are associated with the fibrotic phenotype. The studies in this

thesis may uncover a therapeutic target to attenuate the progression and/or development of pulmonary fibrosis.

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LIST OF ABBREVIATIONS

Abbreviation	Term
AEC	alveolar epithelial cells
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLF	human lung fibroblast cell line
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
IgG	immunoglobulin G
IL-1β	interleukin-1β
IκB	inhibitor of kappa-light-chain-enhancer of activated B cells
IKK	IκB kinase
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	polymerase chain reaction
PEG	polyethylene glycol
PM	provisional matrix
RAC	Ras-related C3 botulinum toxin substrate
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SiRNA	small interfering RNA
SOD	superoxide dismutase
TGF-β	transforming growth factor- β
Th	T helper cells
THP	human acute monocytic leukemia cell line
TNF-α	tumor necrosis factor-α
WT	wide type

CHAPTER 1

INTRODUCTION

1.1 Asbestos-induced pulmonary fibrosis

1.1.1 Pulmonary fibrosis

Pulmonary fibrosis is characterized by aberrant remodeling of injured lung tissue. It is associated with irreversible accumulation of extracellular matrix produced by fibroblasts and myofibroblasts during tissue remodeling, which results in dysfunction and destruction of lung tissue. Normally, lung tissue responds to injury by reversible wound healing. More specifically, damaged lung tissue release cytokines and growth factors that trigger innate and adaptive immune responses that result in tissue repair. However, if the injury is persistent, such as in asbestos exposure, fibrosis may develop.

1.1.2 Asbestos

Asbestos is the major cause of lung fibrosis disease. In the United States, there are more than 200,000 people diagnosed with asbestosis every year, and 2,000 die of asbestos-induced pulmonary fibrosis annually (Roggli, 1990). Since 1850s, asbestos has been used in industry and commerce, but it was not known to be hazardous until l970s. In United States, there are about 1.3 million workers exposed to hazardous levels and 2.3% of male chest films roentgenograms demonstrate asbestos-related lung disease. Vermiculite miners and ship-breakers are in high risk in developing asbestos-induced lung disease (Guidotti et al., 2004). The special issue of *The Lancet* (2011) reported that firefighters exposed to the asbestos at Ground Zero in September 2001 are more likely to develop cancer. Asbestos-induced occupational diseases typically occur after a latency

period of 15-40 years (Cugell and Kamp, 2004). Although strict regulations are now in place, asbestos remains a dangerous toxicant to many workers who are exposed.

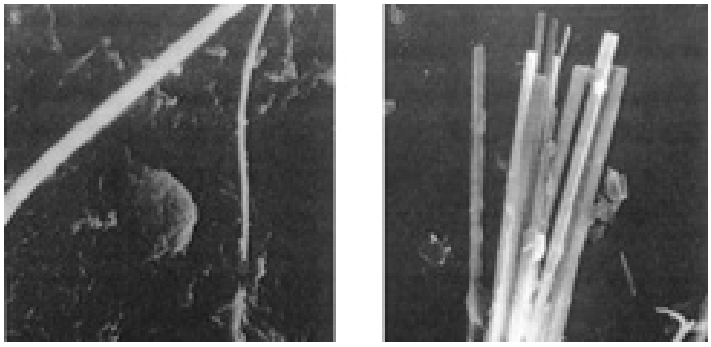
Asbestos-induced lung diseases principally include asbestosis (interstitial fibrosis), pleural disease (effusion, round atelectasis, thickening, and plaques), and malignancies (mesothelioma and lung cancer). The frequency of these diseases is shown in **Table 1.**

Table 1 Ranking and frequency of disease among individuals with 20 or more years of exposure to asbestos

Disease	**Frequency**
Pleural plaques	40,000/100,000
Asbestosis	20,000/100,000
Diffuse pleural thickening	5000/100,000
Lung cancer	2500/100,000
Mesothelioma	1200/100,000

Source: Merchant, J. A. et al.1990. Human epidemiology: a review of fiber type and characteristics in the development of malignant and nonmalignant disease. *Environ Health Perspect.*

Asbestosis is common lung fibrosis caused by inhaled asbestos fibers in lower zones of the lung. It results from deposition and retention of asbestos fibers in terminal bronchioles and lung alveoli. Studies show that the number of asbestos fibers in the alveolar ducts is directly related to development of fibrosis (Miller et al., 1992). Asbestosis patients have insidious onset of dyspnea, nonproductive cough, and wheeze (Brodkin et al., 1993). Smoking can enhance disease development due to reduced clearance of fibers from lung (Barnhart et al., 1990).



Chrysotile **Crocidolite**

Figure 1.1 Asbestos fibers

Source: Internet

Asbestos is a mineral silicate with a Si_2O core and containing iron, magnesium, and calcium (Craighead, 2008; Rolston and Oury, 2004). The hydrated silicate fibers (**Figure 1.1**) have high flexibility, tensile strength, and resistance to heat and acid. They belong to two mineral families (**Table 2**): amphibole and serpentine. Amphibole fibers include crocidolite, amosite, anthophylite, actionolite and tremolite. Chrysotile is the primary serpentine. Because of high carcinogenicity, amphibole asbestos is prohibited for industrial use (Berry and Gibbs, 2008). As a consequence, chrysotile is the most commonly used asbestos in the United States. Fiber length, bio-persistence, and surface reactivity are three main factors to determine the pathological development of lung disease induced by asbestos (Turci et al., 2012). Fiber length can cause "frustrated phagocytosis" by alveolar macrophages, which enhances the generation of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2) (Fubini and Otero, 1999; Kane, 1996).

Table 2 Chemical and morphological properties of asbestos fibers

Family	Characteristics of family	Species	Chemical composition	Specific surface area (m^2/g)
Serpentine	Morphology: curly, bendable	Chrysotile	$Mg_3(Si_2O_5)(OH)_4$	26.8 ± 0.7
	Iron content: ≈1–6%			
	Biopersistence: months			
	Morphological stability: dissolved at pH < 4.5			
Amphibole	Morphology: rigid, robust	Crocidolite*	$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$	8.3 ± 0.5
	Iron content: ≈27–30%	Tremolite	$Ca_2Mg_5Si_8O_{22}(OH)$	NA
	Biopersistence: years	Amosite*	$(Fe,Mg)_7Si_8O_{22}(OH)_2$	5.7 ± 0.3
	Morphological stability: not dissolved at pH < 7	Anthophyllite	$(Mg,Fe)_7Si_8O_{22}(OH)_2$	11.8 ± 10
		Actinolite	$Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_2$	NA

NA not available, * = usual names

Source: Yao, S., DellaVentura, G., Petibois, C. 2010. Analytical characterization of cell-asbestos fiber interactions in lung pathogenesis. *Anal Bioanal Chem*. 397(6):2079-89.

1.2 ROS and H_2O_2

1.2.1 ROS generated by asbestos

As silicate, asbestos fibers in lung can generate free radicals and reactive oxygen species (ROS) in two ways: silica surface generation and cellular activation generation. In water or in presence of H_2O_2, surface silica radicals can form $HO^{\cdot}$ radicals:

$$-SiO^{\cdot} + H_2O \rightarrow -SiOH + HO^{\cdot -} \quad \text{or}$$

$$-SiOO^{\cdot} + H_2O_2 \rightarrow -SiOH + HO^{\cdot} + O_2$$

$$-Si^{+}O_2^{\cdot -} + H_2O_2 \rightarrow -SiOH + HO^{\cdot} + O_2$$

In the presence of iron, the hydroxyl radical can be produced through Fenton mechanism:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + HO^{\cdot}$$

And iron also promotes the formation of $HO^{\cdot}$ from O_2 via Haber-Weiss cycle:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -}$$

$$O_2^{\cdot -} + H_2O \rightarrow HO_2^{\cdot} + OH^{-} \quad \text{or} \; O_2^{\cdot -} + 2H^{+} + e^{-} \rightarrow H_2O_2$$

$$2HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad \text{or} \; O_2^{\cdot -} + H_2O_2 \rightarrow HO^{\cdot} + OH^{-} + O_2$$

Surface ROS generated by particles in the extracelluar space may induce injury to cell components, and activate the cell to produce ROS (Kinnula, 1999). Like surface generation, cellular generation of ROS may also be iron dependent (Knaapen et al., 2004). Phagocytosis of asbestos fibers by alveolar macrophages are results in the generation ROS, especially H_2O_2 (Muthy et al., 2010; He et al., 2011; Osborn-heaford, et al., 2012).

ROS are known to induce cytokine expression in many cell types (Tephly and Carter, 2008). ROS have been shown to differentially regulate the p38 and ERK MAP kinases, which are linked to pro-inflammatory gene expression(Cho et al., 1999; Tephly and Carter, 2007) Transgenic luciferase-reporter mice show that ROS generated by fibrogenic particles mediate pro-inflammatory gene expression (Hubbard et al., 2001). Moreover, studies show that NF-κB, which is a necessary transcription factor for cytokine gene expression, can be activated in asbestos exposure (Sacks et al., 1998). In

addition, ROS generated by asbestos induce apoptosis through DNA damage and death receptor activation (Lim et al., 1999; Iyer et al., 1997).

1.2.2 H_2O_2

H_2O_2 can be generated as by-product of cellular metabolism. H_2O_2 generated by mitochondrial respiration is an important inducer of potent oxidative damage (Giorgio et al., 2007). H_2O_2 can also be generated by enzymes with primary ROS generation function, which including NADPH oxidase family and other oxidases. H_2O_2 local levels can be rapidly elevated in response to injury (Rojkind et al., 2002). Fridovich et al. (1986) found that H_2O_2 is primarily generated from spontaneous dismutation of $O_2\bullet^-$ at a rapid rate, and this reaction can be accelerated by 10^4 fold by superoxide dismutase (SOD). As consequence, high concentrations of H_2O_2 may result in molecular adducts of proteins, lipids, and DNA (Driessens et al., 2009). Thus, to prevent H_2O_2-mediated damage, rapidly increased H_2O_2 needs to be immediately decomposed by antioxidant enzymes, such as catalase and glutathione peroxidase (Leto and Geiszt, 2006).

H_2O_2 mediates oxidative stress in many cell types (Teply and Carter, 2007; Murthy et al., 2009). One study (Kemp and Weitzman, 1999) showed that ROS, including H_2O_2, are associated with cell injury, apoptosis, cell differentiation, persistent inflammation, and fibrogenesis. Studies show that H_2O_2 generated from mitochondrial electron transport chain determines hematopoietic cell differentiation and division (Bedard et al., 2007). In addition, H_2O_2 has been implicated in cellular senescence (Passos and Von Zglinicki, 2006) and apoptosis (Stone and Yang, 2006). H_2O_2 also plays important role in inflammation. In early innate immune response, H_2O_2 kills bacteria through classic ROS respiratory burst (Rada et al., 2008). Recently, new studies

suggest that H_2O_2 can contribute an immune response by quickly recruiting leukocytes (Pase et al., 2012). It has been reported that H_2O_2 plays important role in activation of NF-κB through IκB tyrosine phosphorylation and IKK activation (Schoonbroodt et al., 2000; Yin et al., 2000), which is necessary for pro-inflammatory gene expression. H_2O_2 regulates principal signaling pathways including p38, MARK, and Akt (Hensley et al., 2000; Gabbita et al., 2000). In response to asbestos exposure, monocytes and young macrophages spontaneously generate pro-inflammatory cytokines and ROS, including H_2O_2 (Schwartz et al., 1993; Murthy et al., 2009). Our studies show that H_2O_2 generated from mitochondria has a critical role in the pathogenesis of asbestosis, and inhibition of H_2O_2 attenuates the development of asbestosis (Tephly and Carter, 2007; Murthy et al., 2009).

1.3 Catalase

The predominant role of catalase is the conversion of hydrogen peroxide into water and oxygen (Gaetani et al., 1996). Catalase has been extensively studied since it was first discovered in 1811. However, because of the potent oxidative damage induced by H_2O_2, catalase attracts many investigations into its basic function of eliminating excessive H_2O_2.

Catalase can be classified into three types: mono-functional catalase, bifunctional catalase, and pseudocatalase. Mono-functional catalase is also called typical catalase, which has a tetrameric structure with four equal size subunits containing heme. The molecular weight is about 260kD and is the most common catalase with function of decomposing H_2O_2 into water and oxygen. The bifunctional catalase has the same heme containing tetrameric structure, but additional peroxidase function. Pseudocatalase is

manganese-containing rather than heme-containing enzyme (Peter et al., 2000). The rigid and stable tetrameric structure gives catalase broad pH endurance and high thermal resistance, which ensures its function in different circumstances. It can reduce millions of H_2O_2 molecules into water and oxygen per second (Young and Woodside, 2001). The catalase structure and two-stage reaction is shown in **Figure 1.3**.

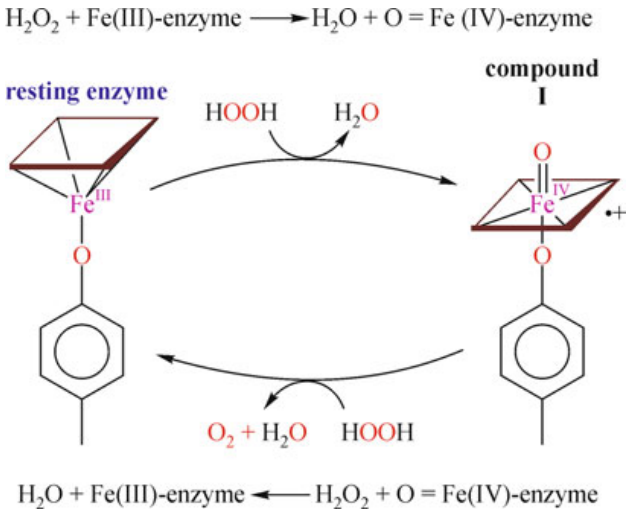


Figure 1.3 Catalase structure and mechanism

Source: Anderson et al. 1991. *Structure and Bonding*.

By decomposing H_2O_2, especially produced in mitochondria, catalase functions to maintain the redox balance in cells. Excessive H_2O_2 is harmful to cell functions (Zamocky et al., 2008). Studies show that over expressed catalase in transgenic mice prevents mitochondrial insults caused by H_2O_2 and extends life span by decreasing DNA adducts (Schiner et al., 2005). Odajima et al. (2010) found that in human lung fibrosis and in bleomycin-induced mouse lung injury, diminished catalase worsened the

development of pulmonary fibrosis. One study showed that catalase prevents H_2O_2 induced damage to mitochondrial membranes, which can result in loss in membrane potential, leakage of cytochrome *c*, and activation of apoptosis (Nomura et al., 1999). Studies also showed that targeting catalase to the mitochondria in lung epithelial cells protected the cell from H_2O_2-induced apoptosis (Arita et al., 2006). Taken together, these observations suggest that catalase may attenuate lung injury by decreasing H_2O_2 levels.

1.4 Cellular response to oxidative stress

1.4.1 Alveolar epithelial cells

Asbestos-induced lung injury starts with damage of alveolar epithelial cells (AEC). There are two types alveolar epithelial cells. Type I (squamous alveolar) cells cover 90% of alveolar surface and form the structure of alveolar wall. Type II (great alveolar) cells produce surfactant to decrease the aqueous surface tension, which allows the membranes to be more permeable and maintains alveolar structure. This increases gas exchange in the alveolar spaces. Type II cells can differentiate to type I cells. Alveolar epithelial cells react to various insults by releasing cytokines and free radicals to activate immune response and coagulation pathway (Wynn and Ramalingam, 2012). The coordinated responses result in fibroblast activation for wound repair and homeostasis restoration. AEC apoptosis is an important event in the pathogenesis of asbestosis and is associated with DNA damage and p53 activation (Panduri et al., 2006).

1.4.2 Alveolar Macrophages

Alveolar macrophages are large mononuclear phagocytic cells. Traditionally, these cells are viewed as the first responder to injury by initiating phagocytosis, antigen presentation, Th1 cytotoxicity, and induction of humoral immunity (Geissmann et al.,

2010). The precursors of macrophages are monocytes. Monocytes originate from committed hematopoietic stem cells in the bone marrow, and are then released into the bloodstream. Monocytes differentiate into macrophages or dendritic cells when they migrate into tissue. There are two groups of macrophages: resident macrophages and recruited macrophages. Resident alveolar macrophages clear asbestos by phagocytosis. This response to asbestos results in the recruitment of blood monocytes. Alveolar macrophages initiate the inflammatory reaction and generate ROS (Murthy et al, 2010). H_2O_2 generated from the macrophage mitochondria has a critical role in the pathogenesis of asbestosis, which suggests that abrogation of mitochondrial oxidative stress can attenuate the development of fibrosis (He et al., 2010; Osborn-Heaford et al., 2012).

1.4.3 Monocytes

Monocytes can differentiate into macrophages (Geissmann et al., 2010). Like macrophages, monocytes play important role in immune response during pulmonary fibrosis. In initial lung injury, damaged epithelial cells and endothelial cell activate coagulation cascade and cytokines to recruit monocytes and neutrophils as part of the innate immune response (Kreisel et al., 2010). The “frustrated phagocytosis” of asbestos fibers by alveolar macrophages can result in apoptosis (Osborn-Heaford et al., 2012). The depletion of macrophages is alleviated by the recruitment of monocytes. Spurzem et al. (1987) reported that the majority of cells in the BAL fluid in chronic lung disease, including pulmonary fibrosis are monocytes and young macrophages. This observation was further confirmed in a study showing the bronchoalveolar (BAL) cells obtained from asbestosis patients are younger or monocytes-like (Rom et al. 1985). In addition, alveolar macrophages from patients with asbestosis are known to function differently than

macrophages from normal subjects (Hance et al., 1985). Taken together, monocytes play a crucial role in asbestos-induced lung injury and fibrosis development.

1.4.4 Fibroblasts

Fibroblasts produce collagen to form tissue structure. Aberrant collagen deposition occurs in fibrosis development. Specifically, in response to lung injury, fibroblasts are transformed to myofibroblasts, which produce collagen for wound repair and homeostasis restoration. However, in the setting persistent injury, such as caused by asbestos exposure, a continual activation of myofibroblasts results in production of excessive collagen that leads to fibrosis development (Vuorio et al., 1989).

1.4.5 Pro-inflammatory mediators

Cytokines are secreted by innate immune cells (including macrophages, neutrophils, eosinophils and mast cells) during lung disease development. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are two pro-inflammatory cytokines that are expressed in response to lung injury. Clinical trials have been initiated to evaluate effects of TNF-α inhibitor therapy for pulmonary fibrosis and other fibrotic diseases (Raghu et al., 2008). In addition, TNF-α receptor knockout mice exposed to silica or asbestos are protected from developing fibrosis (Nawroth et al., 2010; Jones et al., 2009), which suggests that TNF-α is important in fibrosis development.

Prior studies from our lab show that p38 MAP kinase activation regulates asbestos-induced TNF-α expression in monocytes (Carter et al., 2004). We have also shown that the GTPase binding protein, Rac1, modulates TNF-α gene expression (Murthy et al., 2009). Additionally, we have observed that in alveolar macrophages, O_2^- generation mediates chemokine expression after TNF-α stimulation in an ERK-dependent

manner (Tephly and Carter 2008). However, the regulation of collagen production by TNF-α is controversial. Kalluri et al. (2006) reported that collagen expression is differentially modulated by TNF-α. More specifically, TNF-α has been shown to both increase and decrease collagen production. Based on these observations, TNF-α has an important role fibrosis development; however, the relationship between oxidative stress, TNF-α, and the fibrotic phenotype is unknown.

Like TNF-α, IL-1β is another pro-inflammatory cytokine in that as been investigated in pulmonary fibrosis. IL-1β has been shown to have pro-fibrotic activity (Gasse et al., 2007), and IL-1β knockout mice have attenuated bleomycin-induced pulmonary fibrosis compared to WT mice (Bujak et al., 2009). In addition, IL-1β, in combination with transforming growth factor- β (TGF-β), regulates myofibroblast activation. In aggregate, TNF-α and IL-1β are critical pro-inflammatory cytokines in the immune response to lung injury suggesting that they may have a role in fibrosis development.

1.5 Relationship between oxidative stress, pro-inflammatory cytokines, and pulmonary fibrosis.

Previous studies in our lab have linked mitochondrial oxidative stress to the development of pulmonary fibrosis (Murthy et al., 2010; He et al., 2011; Osborn-Heaford et al., 2012). In addition, we showed that fibroblasts produce less collagen when cultured in conditioned media obtained from macrophages with low H_2O_2 generation (Rac1 null and Cu,Zn-SOD$^{-/-}$) compared to fibroblasts cultured in conditioned media from WT macrophages. In contrast to the traditional dogma, we have since found that the mice protected from pulmonary fibrosis had increased expression of pro-inflammatory

cytokines in BAL fluid and in alveolar macrophages. Thus, these observations suggest that there is an inverse relationship between oxidative stress and pro-inflammatory cytokine gene expression.

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

In response to asbestos, macrophages generate ROS, especially H_2O_2, and secrete pro-inflammatory cytokines, such as TNF-α and IL-1β, to activate fibroblasts, resulting in collagen production and pulmonary fibrosis formation. H_2O_2 plays important role in fibrosis development. Catalase can decompose H_2O_2 into water and oxygen. Thus the hypothesis is that catalase regulates asbestos-induced pro-inflammatory cytokine production and development of fibrosis.

2.2 Study objectives

2.2.1 Investigation of TNF-α function

Objective 1: TNF-α is a pro-inflammatory cytokine factor produced by inflammatory cell and modulates collagen expression.

During initial lung injury, damaged epithelia and endothelia cells release cytokines, triggering an innate immune response. Stimulated by IL-1β, monocytes produce TNF-α, which modulates collagen production by fibroblasts. To simulate this, monocytes are treated with IL-1β in different doses for 24h and asbestos is added for the last 4h. The conditioned medium will be analyzed for TNF-α, and applied to fibroblasts for 24h incubation. Collagen produced by fibroblasts will be determined. The experiment gives relationship between TNF-α and collagen, which will investigate the role of pro-inflammatory cytokines in fibroblast collagen production.

Objective 2: TNF-α modulates fibrosis development

The effect of TNF-α on collagen production depends on TNF-α binding to its receptor on the fibroblast cell. If the binding is blocked, the role of TNF-α on collagen production may be better defined. A TNF-α receptor-1 siRNA was designed to test the mediator function of TNF-α. After 48-72h TNF-α receptor siRNA transfection of fibroblast, TNF-α receptor-1 expression will be analyzed by Western blot to determine whether it has been knocked down. A successful condition of TNF-α siRNA receptor-1 transfection will be defined. After such transfection, the fibroblasts are incubated with conditioned medium of monocytes and collagen production will be determined.

2.2.2 Investigation of catalase function

Objective 1: Catalase alters TNF-α and IL-1β in monocytes and modulates expression of collagen in fibroblasts

Asbestos exposure can result in an inflammatory response. Acute injury effect can be observed through monocytes experiments. Monocytes will be treated with catalase for 24 h followed by asbestos exposure for 4 h. TNF-α and IL-1β mRNA and protein secreted into the medium will be determined. The conditioned medium adjusted with equal protein contents will be applied to fibroblasts. After 24hr incubation, medium is analyzed by immunoblot for collagen expression, as a measurement of fibrosis development.

Objective 2: *In vivo*, catalase attenuates lung fibrosis in mice while increasing pro-inflammatory cytokines.

To investigate the biological relevance asbestos-induced fibrosis, we have designed an *in vivo* study utilizing mice exposed to asbestos. As above mentioned,

asbestos trapped in alveoli maintains constant irritation by frustrating resident macrophages and recruited monocytes, resulting in persistent fibroblast activation and fibrosis formation. After asbestos exposure by intratracheally administration, mice are treated with catalase intratracheally daily for 20 days. Mice are euthanized at day 21. BAL is obtained for cell differentiation and cytokine expression and lung tissue is harvested for hydroxyproline test and collagen histology staining.

Objective 3: Catalase effects on cell viability

TNF-α and IL-1β can induce cell death, and asbestos is known to be cytotoxic to cells. However, it remains unknown how catalase affects cell viability of monocytes and fibroblasts. Cell death occurs either by apoptosis or necrosis, which is associated with plasma membrane permeability (Bonfoco et al., 1995). As a soluble enzyme, lactate dehydrogenase (LDH) is released to the surrounding medium upon cell damage. By measuring LDH in medium of monocyte and fibroblast, cell viability will be evaluated.

Objective 4: Catalase effects on p38 and ERK MAP kinases

MAP kinases transfer signals from the cell surface to the nucleus through phosphorylation. Studies show that MAP kinases are activated by asbestos and H_2O_2 is a mediator of MAP activation. Monocytes will be treated with catalase for 24h and added asbestos for the last 4 h. The whole cell lysates will be analyzed for p38 and ERK and phosphorylated p38 and ERK. In both monocytes and mice experiments, heat-denatured catalase will be investigated in parallel with normal catalase.

CHARPTER 3

MATERIALS AND METHODS

3.1 Materials and reagents

Chrysotile asbestos was provided by the North American Insulation Manufacturers Association Fiber Repository. Catalase was purchased from Worthington Biochemical (Lakewood, NJ). Catalase-polyethylene glycol (PEG-catalase) was purchased from Sigma. (St. Louis, MO). Rabbit polyclonal IgG to ERK2, mouse monoclonal IgG to p-ERK, rabbit polyclonal IgG to p-38, rabbit polyclonal IgG to TNF-α receptor-1, mouse monoclonal IgG to p-ERK, rabbit polyclonal IgG to collagen, goat anti-mouse IgG-horseradish peroxidase (HRP), goat anti-rabbit IgG-horseradish peroxidase (HRP), TNF-α receptor-1 SiRNA from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibodies β-actin from Upstate Biotechnology, Millipore (Billerica, MA) and Sigma (St. Louis, MO); Rabbit monoclonal IgG to phospho-p38 MARK from Cell Signaling Technology, Inc. (Danvers, MA); TNF-α, IL-1β ELISA kits from R & D Systems (Minneapolis, MN); LDH Cytotoxicity Assay Kit from Cayman Chemical Company (Ann Arbor, MI).

3.2 Cells

The human monocyte THP-1 cell line (ATCC, Manassas, VA) were maintained in RPMI 1640 containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 2 mM L-glutamine, 10% fetal bovine serum, and gentamicin. The human lung fibroblast HLF-1 cell line (ATCC, Manassas, VA) was maintained in DMEM containing 4.5 g/l glucose, L-glutamine, sodium pyruvate, 10% fetal bovine serum and penicillin/streptomycin.

3.3 Mice

Wild-type (WT) C57BL/6 mice were used in these studies, and all protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. A bolus dose of 100 μg of chrysotile asbestos in 50 μl of normal saline was instilled intratracheally to 6- to 24-wk-old mice after 2–5 min of anesthesia with 3% isoflurane via a precision Fortec vaporizer (Cyprane, Keighley, UK). Then mice were divided in three groups and administered intratracheally with water, catalase and heat denatured (95°C, 30 min) catalase respectively, daily for 21 days. On day 21 the mice were euthanized with an overdose of isoflurane, and bronchoalveolar lavage (BAL) was performed. BAL cells were used for determination of total and differential cell number. BAL fluid was used for determination of cytokine and lung tissue was used for hydroxyproline concentrations. The lungs were removed and stained for collagen fibers using Masson's trichrome.

3.4 PEG-catalase treatment

THP-1 Cells were treated with polyethylene glycol catalase of 100 units/ml in 0.5% newborn calf serum medium for totally 24 hours. Cells were exposed to chrysotile asbestos for the last 4 hours.

3.5 Hydroxyproline determination

Referred to Begin, R. (2001), the pulmonary fibrosis is associated with increased hydroxyproline levels. A tip of lobe (about 50mg) of mouse lung was cut off into a glass bottle and dried in 200°C oven. After weight recorded, the solid tissue was digested for

24 h at 110°C with hydrochloric acid at a final concentration of 6 N, and hydroxyproline content was determined as previously described (Englert et al., 2008).

3.6 Histological scoring

Sections of right lungs that were stained with Masson's trichrome were scored for fibrosis by a pathologist who was blinded to the treatment groups. Separate fields associated with aggregates of inflammatory cells in each section were scored as follows: 0 for normal, 1 for peribronchial fibrosis, 2 for parenchymal fibrosis, 3 for peribronchial and parenchymal fibrosis, and 4 for widespread fibrosis.

3.7 Cytokine determinations

TNF-α, IL-1β in conditioned media of cell culture experiments and BAL fluid were measured by cytokine-specific ELISA Duo Set kits. BAL cytokines were normalized to total protein levels that were determined using the Micro-BCA kit (Pierce, Rockford, IL).

3.8 Cell viability

Lactate dehydrogenase (LDH) is released to the surrounding medium upon cell damage. Cell viability was measured by LDH Cytotoxicity Assay Kit (Cayman Chemical Company, Ann Arbor, MI).

3.9 Quantitative real-time PCR

Total RNA from experimental cells was isolated by TRIzol. Reverse transcription for cDNA was processed with reverse transcriptase kit Iscript (Bio-Rad Laboratories, Hercules, CA). Human TNF-α and IL-1β and HPRT mRNA transcripts were determined by quantitative real-time PCR using SYBR Green (Bio-Rad Laboratories) and respective

primers on an IQ5 real-time PCR machine (Bio-Rad Laboratories). The following primer sets were used:

TNF-α

5′-CCACATCTCCCTCCAGAAA-3′ (forward)

5′-CACTTGGTGGTTTGCTACGA-3′ (reverse)

IL-β1

5′-AACAGGCTGCTCTGGGATTCTCTT-3′ (forward)

5′-ATTTCACTGGCGAGCTCAGGTACT-3′ (reverse)

HPRT

5′-AGCCCTGGCGTCGTGATTAGTGA-3′ (forward)

5′-TGTCCCCTGTTGACTGGTCATTACA-3′ (reverse)

Data were calculated by the cycle threshold ($\Delta\Delta C_T$) method.

3.10 Western blot analysis

Cell lysates were made by using cell lysis buffer. Cell lysates or cell culture supernatants samples were separated by SDS-PAGE. Immunoblot analyses were performed with designated primary antibodies and corresponding secondary antibody-HRP conjugates.

3.11 TNF-α receptor-1 RNA interference(siRNA)

HLF-1 human lung fibroblast cells were plated into to 24-well plate and incubated at 37°C with 5% CO_2 overnight to 60% confluence. 1uM siRNA and 4% (V/V) Dharma FECT 4 transfection agent were prepared by gently pipetting in serum free DMEM medium then incubated for 5 minutes at room temperature. Two contents were mixed

carefully and incubated for 20 minutes at room temperature. Add sufficient antibiotic-free complete medium to the mix to a final volume, in which siRNA was 100nm and Dharma FECT 4 was1% (V/V). Remove culture medium from the wells of 24-well plate and add 300 ul of the transfection medium to each well. Incubate cells at 37°C in 5% CO_2 for 48hrs or 72hrs. Remove transfection medium and add THP1 cell conditioned medium to the wells. Incubate at 37°C in 5% CO_2 for 24hrs. Supernatant was for collagen while cell lysate for TNF-α receptor 1 western blot analyses.

3.12 Statistical analysis

Statistical comparisons were performed using an unpaired, one-tailed *t*-test. Values are means ± SE. $P < 0.05$ was considered to be significant.

CHAPTER 4

RESULTS

4.1 IL-1β increases TNF-α in macrophages and decreases fibroblast collagen production.

Because pro-inflammatory cytokines have both a positive and negative effect on collagen production, we investigated if macrophage-derived cytokines modulated secretion of collagen from fibroblasts. After lung injury, damaged epithelia and endothelia cells release cytokines to trigger immune response. The recruited monocytes and macrophages produce growth factors and cytokines to activate fibroblasts, which produce collagen. To simulate this, macrophages were stimulated by IL-1β at two concentrations doses (100 pg/ml, 1000 pg/ml), and TNF-α expression was determined. The low dose of IL-1β (100 pg/ml) was no different from control, while both chrysotile and IL-1β (1000 pg/ml) increased TNF-α significantly (**Figure 4.1**). This was further increased when chrysotile and IL-1β were combined.

To determine the effect of TNF-α on collagen production, HLF-1 cells were cultured with the conditioned media for 24 hr. Pro-collagen and collagen production was measured in the media, and cells cultured with higher levels of TNF-α resulted in a greater decrease in pro-collagen (**Figure 4.2**). Pro-collagen is precursor of collagen, generated in fibroblasts and ready to be cleaved to form collagen. We found that high dose (1000 pg/ml) of IL-1β stimulated higher expression of TNF-α than low dose (100 pg/ml), and chrysotile augmented the TNF-α expression when combined with IL-1β. These results suggest that there is an inverse relationship of macrophage-derived TNF-α production and collagen secretion by fibroblasts.

4.2 Catalase increases asbestos-induced TNF-α and IL-1β expression and decreases collagen production.

H_2O_2 induced by asbestos plays important role in pulmonary fibrosis development. It is not clear whether catalase can attenuate fibrosis by converting H_2O_2 into water and oxygen. THP-1 macrophages were treated with PEG or PEG-catalase overnight, and cells were exposed to chrysotile asbestos for the last 4h. TNF-α and IL-1β secretion was determined (**Figure 4.3**). Whole cell RNA was isolated by TRIzol for mRNA test by real-time PCR (**Figure 4.4**), but there was not statistical difference with asbestos exposure. In contrast, asbestos exposure in the presence of heat-denatured catalase increased TNF-α and IL-1β compared to heat-denatured catalase alone. Catalase increased TNF-α and IL-1β in both the presence and absence of asbestos. In addition, the protein expression pattern was identical to that of mRNA, indicating consensus of TNF-α and IL-1β production between the transcriptional and translational level.

To determine the effect of pro-inflammatory cytokines on collagen production, HLF-1 cells were cultured with the conditioned media for 24 hr. Pro-collagen and collagen production was measured in the media, and cells cultured with higher levels of TNF-α and IL-1β had a greater decrease in pro-collagen (**Figure 4.5**).

We tested catalase enzymatic activity and protein concentration of 1 mg/ml denominated Sigma PEG-catalase product in regular and heat-denatured status. To determine the activity of PEG-catalase and heat-denatured catalase, an enzymatic assay was performed in the University of Iowa Free Radical and Antioxidant Enzyme Core. We found that the activity remaining in the heat-denatured catalase was 8.1% of the

PEG-catalase stock (**Table 3**). We evaluated if the lower catalase activity of the heat-denatured catalase had a similar effect in modulating pro-inflammatory cytokine production. In the presence of asbestos, heat-denatured catalase increased TNF-α and IL-1β protein expression was less than PEG-catalase (**Figure 4.3**). However, the level of TNF-α and IL-1β gene expression were higher than in cells treated with PEG-catalase (**Figure 4.4**). In addition, heat-denatured catalase decreased collagen production to a greater extent compared to PEG-catalase (**Figure 4.5**). It will be important in future experiments to inactivate catalase completely in similar experiments. Taken together, the results show that catalase increases TNF-α or IL-1β in macrophages and decreases fibroblast collagen production. These observations reveal an inverse relationship between catalase and pro-inflammatory gene expression.

Table 3 Catalase activity and protein concentration

Issue	**Method**	**PEG-Catalase**	**Heat-denatured PEG-Catalase**
Enzymatic Activity (k unit)	H_2O_2 Absorbance	122.447	1.305
Protein Concentration (mg/ml)	Micro BCA	2.892	0.378
Activity/ Protein (k units/mg)		42.339	3.452

4.3 Catalase has no effect on cell viability

It is well known that pro-inflammatory cytokines can induce cell death, and asbestos has cytotoxicity. Cell death occurs either by apoptosis or necrosis, which are associated with plasma membrane permeability (Bonfoco et al., 1995). We determined if cell viability had a role in cytokine expression in our model. As a soluble enzyme, lactate dehydrogenase (LDH) is released to the surrounding medium upon cell damage. THP-1 macrophages were treated with PEG-catalase overnight and then exposed to chrysotile asbestos for 4h. HLF-1 cells were cultured in the conditioned media obtained from macrophages for 24h and LDH was measured (**Figure 4.6**). In THP-1 media, asbestos induced an increase in LDH, and catalase had no effect on the asbestos-induced cytotoxicity. In media from HLF-1 cells, LDH was increased in media obtained from asbestos-exposed THP-1 cells. Interestingly, LDH was even greater in HLF-1 cells cultured with THP-1 media treated with heat-denatured catalase. In aggregate, asbestos causes increased cell death of macrophages and fibroblasts; however, catalase has no effect on cell viability.

4.4 TNF-α is a pro-inflammatory cytokine associated with certain fibrotic conditions.

Previous studies showed that TNF-α is a key mediator in pulmonary fibrosis (Piguet et al., 1989), but these findings have not been able to be reproduced. The data in this thesis shows that TNF-α and collagen production are inversely related. Based on these findings, we investigated whether there was a direct effect of TNF-α on fibroblasts that resulted in the down-regulation of collagen production. We hypothesized that TNF-α induced apoptosis, necrosis, or cellular senescence in lung fibroblasts. The TNF-α receptor-1 has been associated with the induction of apoptosis (Eum and Billiar, 2011), so we knocked down TNF-α receptor 1 in fibroblasts to determine if it reversed the effect of TNF-α on fibroblast collagen production. **Figure 4.5** showed that TNF-α receptor 1 in fibroblast is blocked by siRNA. HLF-1 fibroblasts cells were transfected with a scrambled or TNF-α receptor 1 siRNA for 48h or 72h. TNF-α receptor 1 in fibroblasts was determined for its knock down by siRNA. Compared with scrambled control, TNF-α receptor 1 siRNA decreased TNF-α receptor 1 expression most significantly at 72 h after transfection. Based on this successful knock down, the function of TNF-α in fibroblast collagen production was investigated. HLF-1 fibroblasts were transfected with scrambled or TNF-α receptor 1 siRNA for 72 h and then cultured with conditioned media obtained from THP-1 cells treated by PEG or PEG-catalase in the presence or absence of asbestos. After 24h, the media from fibroblasts were collected and pro-collagen and collagen secretion into the media was determined by immunoblot analysis (**Figure 4.7**). For scrambled siRNA transfected fibroblasts, the expression of pro-collagen and collagen was similar as that shown in PEG-catalase treatment experiment (**Figure 4.5**). In

contrast, the fibroblasts transfected with TNF-α receptor 1 siRNA, had significantly increased pro-collagen and collagen expression compared to the cells expressing the scrambled siRNA (**Figure 4.8**). The densitometry analysis of three experiments is shown (**Figure 4.9**). These observations suggest that macrophage TNF-α production is inversely related to fibroblast collagen production. In the future, the immunoprecipitation of TNF-α from macrophage conditioned media or knock down of TNF-α in macrophages with siRNA should be performed to confirm these findings. In addition, the mechanism—apoptosis, necrosis, and/or cellular senescence—of TNF-α regulation on collagen production will be investigated.

4.5 Catalase attenuates asbestos induced lung fibrosis in mice

To show that our *in vitro* results were biologically relevant, we investigated asbestos-induced pulmonary fibrosis in an animal model. WT C57BL/6 mice were intratracheally administered with 100ug/mouse chrysotile asbestos. For 20 days, they were administered with either water, catalase, or heat-denatured catalase daily. On day 21, mice were euthanized. BAL was collected for cell differentiation analysis, cytokine levels, and collagen by immunoblot analysis. Lung tissues were stained for trichrome and hydroxyproline concentration was determined. We found that pro-collagen production was present in all water treated mice and one treated with heat-denatured catalase (**Figure 4.10**). In contrast, no pro-collagen was seen in catalase-treated group. The collagen expression in all three mice in the catalase group showed a decrease in collagen compared with water control and the heat-denatured catalase. This suggests that catalase attenuates pulmonary fibrosis. This was also evaluated by the Masson's trichrome staining **(Figure 4.11**). For water control mice, the positive collagen staining indicates fibrosis formation in lung. The staining in lungs of catalase treated mice was nearly absent, which provides additional evidence that catalase attenuates fibrosis development. To confirm this biochemically, hydroxyproline concentration in lung tissue was determined. In **Figure 4.12,** hydroxyproline concentration was decreased in catalase-treated mice, suggesting that catalase prevents development of lung fibrosis. Moreover, compared with water control, catalase treated mice showed a significant increase of TNF-α in BAL fluid (**Figure 4.13**). Taken together, the *in vivo* experiments show that catalase attenuates asbestos-induced pulmonary fibrosis while increasing pro-inflammatory cytokines in mice.

4.6 Catalase inhibits asbestos-induced activation of p38 and ERK MAP kinase.

The p38 and ERK MAP kinases are second messengers for transferring signal from the cell surface to the nucleus. The signaling transduction is via phosphorylation of p38 and ERK. Studies indicate that MAP kinases are activated by asbestos (Carter et al., 2004; Tephly and Carter, 2007; Tephly and Carter, 2008). As can been seen in **Figure 4.14**, both phosphorylated p38 and ERK were increased, indicating activation of p38 and ERK kinases by asbestos. H_2O_2 has been shown to mediate MAP activation. It can be seen that both phosphorylated p38 and ERK are decreased by PEG-catalase pre-treatment before asbestos exposure for 4 h. Our previous studies show that overexpressing catalase decreases p38 activation and TNF-α production in activation in monocytes (Tephly and Carter, 2007). In the studies in this thesis, p38 MAP kinase activation was decreased by catalase treatment, but the PEG-catalase increased TNF-α after asbestos exposure. For catalase treatment, actually the asbestos treated phosphorylated ERK was even lower than that of no asbestos and the phosphorylated p38 remained without change. It can be explained that catalase inhibits activation of p38 and decreases activation of ERK induced by asbestos. The heat-denatured catalase, which has 8.1% catalase activity inhibited p38 MAP kinase to a greater extent, but less on ERK. Some difference has been observed on ERK activation in a similar experiment utilizing a catalase delivered by Adv-vector (**Figure 4.15**), in which phosphorylated ERK was increased. These differences may be due to cellular localization of the enzyme, i.e., PEG-catalase sticking to the interior surface of the cell membrane while the Adv-vector expressed catalase may be in the cytoplasm. The localization should be determined in future experiments.

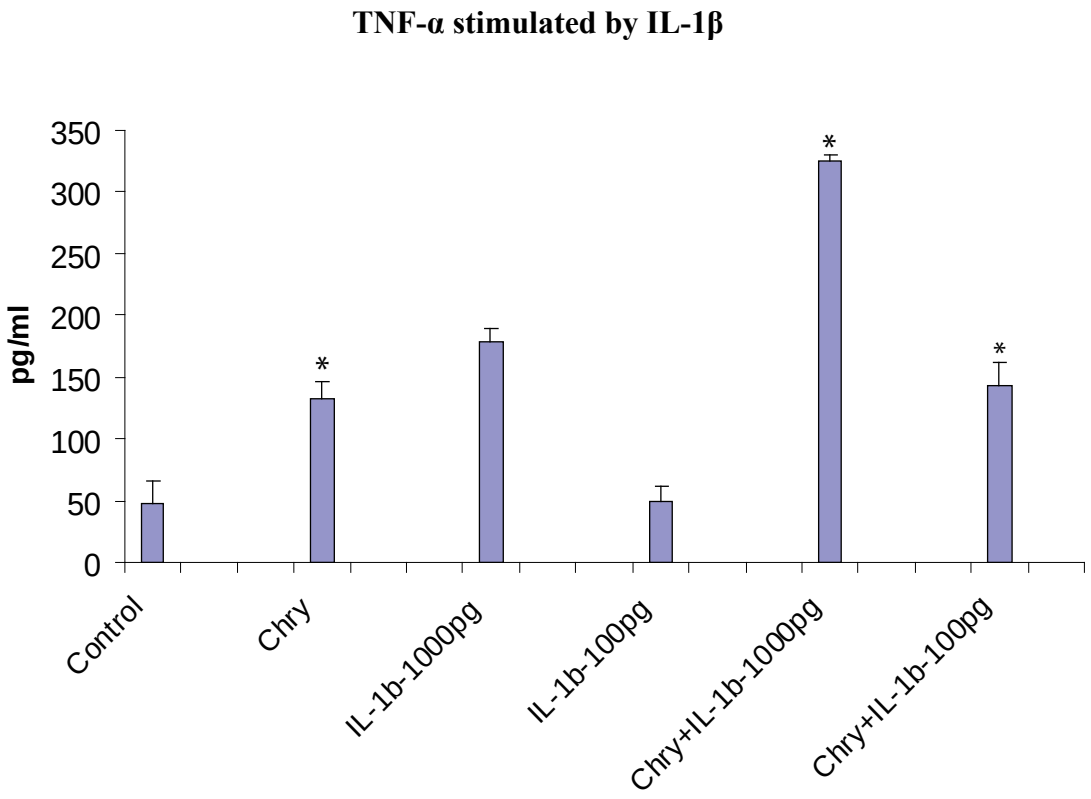


Figure 4.1 THP-1 cells produce TNF-α in response to chrysotile exposure and IL-1β stimulation.
THP-1 cells were treated with IL-1β (100 pg/ml, 1000 pg/ml) for 24h. For the last 4h, cells were exposed to chrysotile(Chry) asbestos (10μg/cm^2). TNF-α was measured in conditioned media by ELISA. n= 3, *, p<0.05, chrysotile (+) versus (-) in each condition.

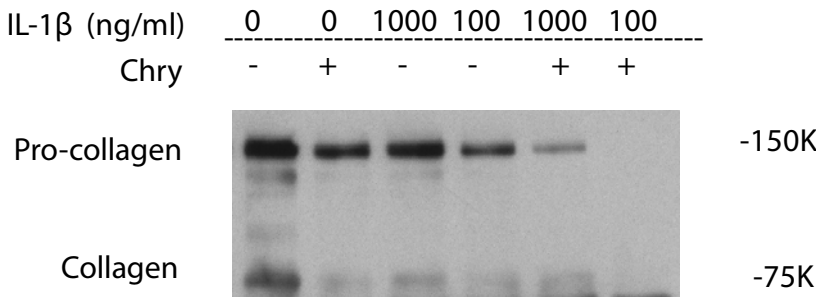


Figure 4.2 IL-1β decreases collagen secretion from HLF-1 cells.
HLF-1 cells were cultured with conditioned media from THP-1 treated with IL-1 β in the presence or absence of chrysotile. Collagen secretion was determined in 20 μg of media by immunoblot analysis. This experiment was repeated 3 times.

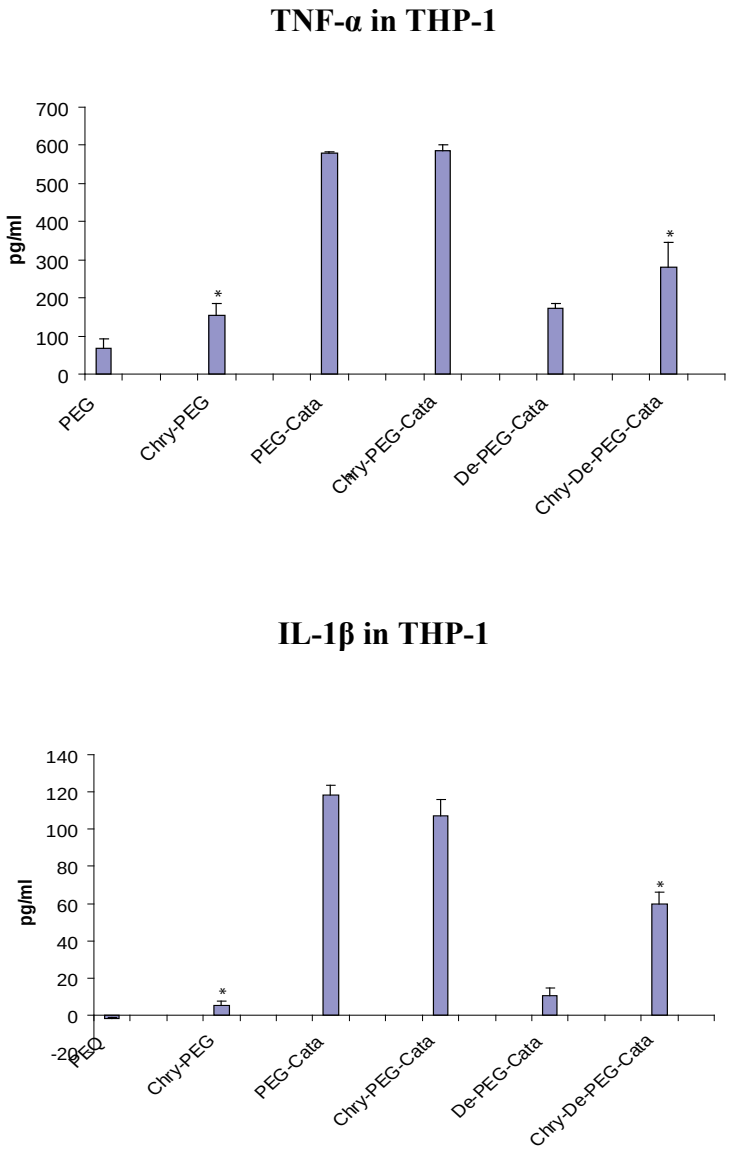


Figure 4.3 Catalase increases TNF-α and IL-1β in THP-1 cells.
THP-1 cells were treated with PEG-catalase (100 units/ml) or heat-denatured (95°C, 30 min) PEG-catalase for 24h and then exposed to chrysotile asbestos (10μg/cm^2) for 4h. Conditioned media were analyzed for TNF-α and IL-1β expression by ELISA. n= 3, *, p<0.05, chrysotile (+) versus (-) in each condition.

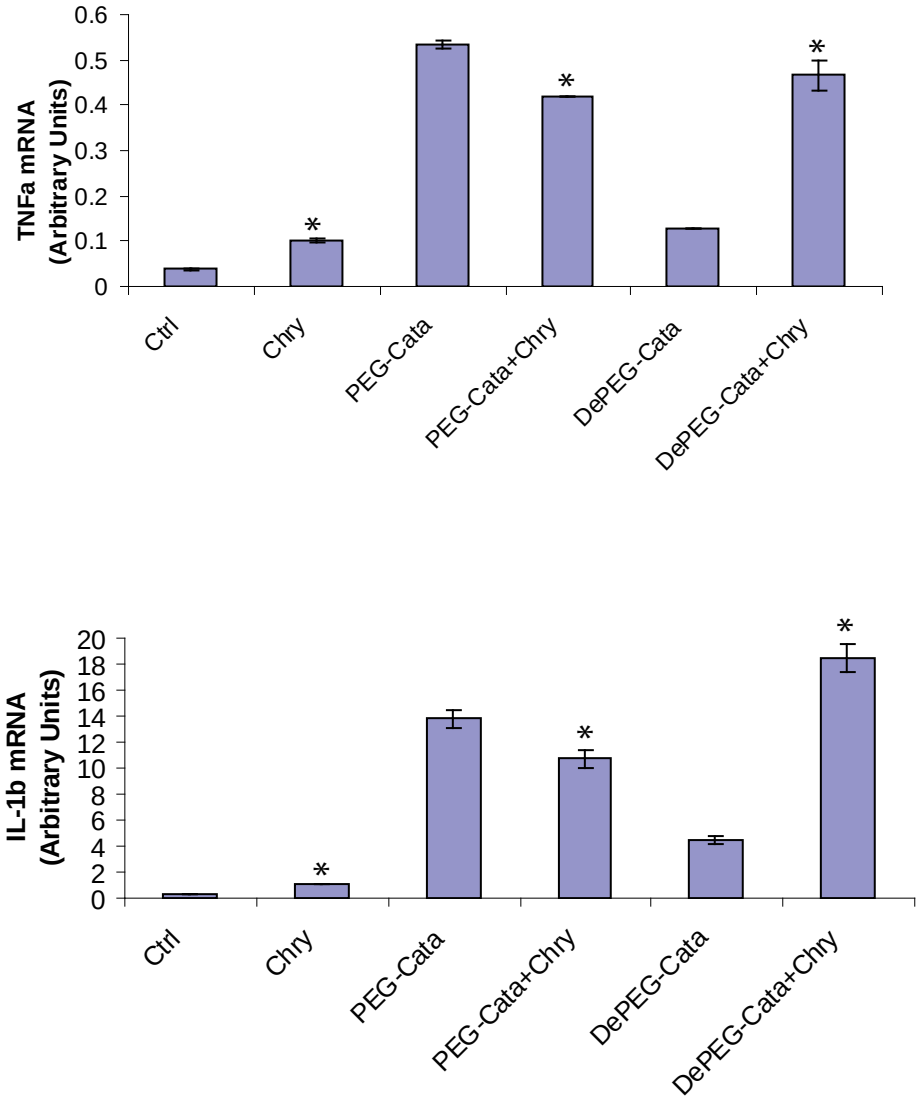


Figure 4.4 Catalase increased TNF-α and IL-1β gene expression.
THP-1 cells were treated with PEG-catalase (100 units/ml) or heat-denatured (95°C, 30 min) PEG-catalase for 24h and then exposed to chrysotile asbestos (10μg/cm^2) for 4h. Total RNA from THP-1 cells was isolated by TRI-zol and subjected to reverse transcription. TNF-α, IL-1β, and HPRT mRNA transcripts were determined by quantitative real-time PCR. n= 3, *, p<0.05, chrysotile (+) versus (-) in each condition.

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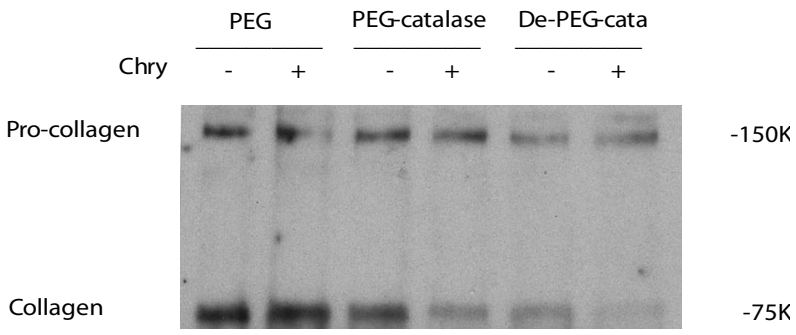

Figure 4.5 HLF-1 collagen production is decreased by conditioned media from THP-1 cells treated with PEG-catalase.
HLF-1 cells were incubated in THP-1 conditioned medium treated with PEG-catalase (100 units/ml) for 24h. Media were analyzed for pro-collagen and collagen by immunoblot analysis. This experiment was repeated 4 times.

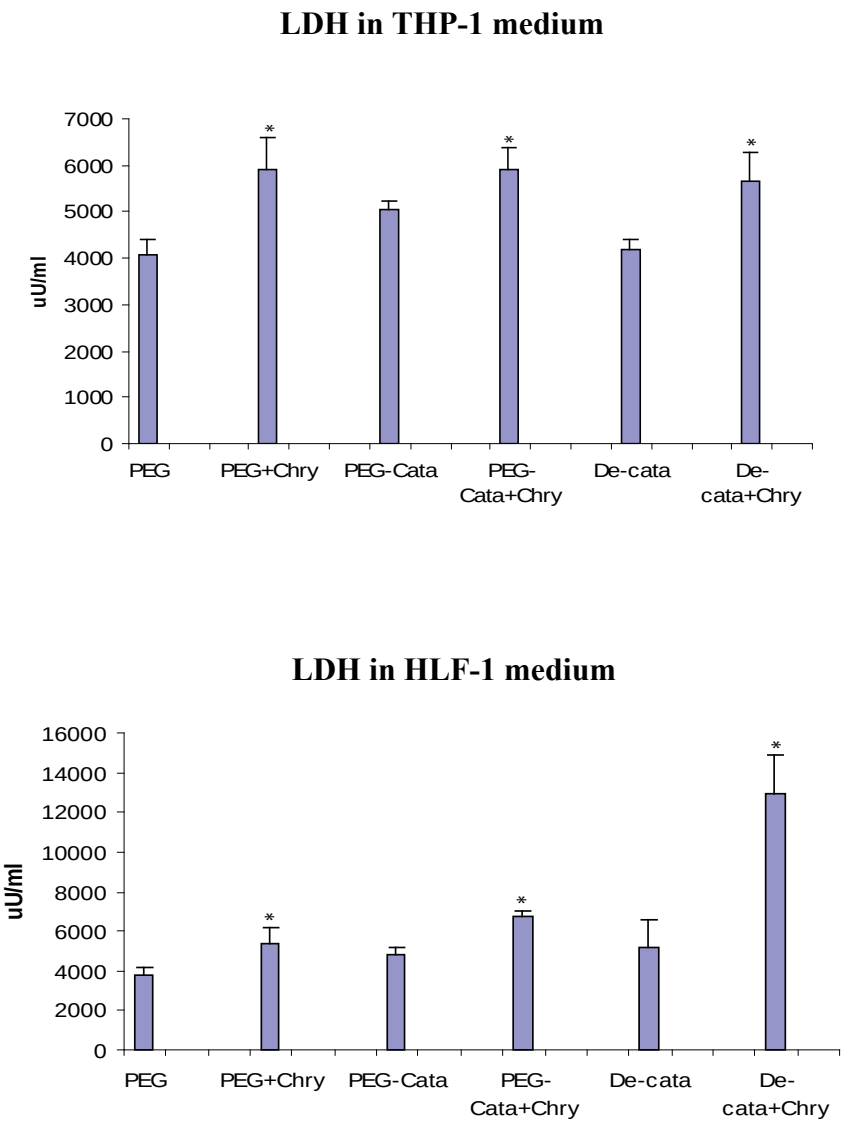

Figure 4.6 Asbestos exposure induces cytotoxicity to macrophages and fibroblasts. THP-1 cells were treated with PEG-catalase (100 units/ml) or heat-denatured (95°C, 30 min) PEG-catalase for 24h. For the last 4h, cells were exposed to chrysotile asbestos (10μg/cm^2). HLF-1 cells were cultured in THP-1 conditioned media for 24h. THP-1 conditioned media and HLF-1 medium were analyzed for LDH concentration. n= 3, *, p<0.05, chrysotile (+) versus (-) in each condition.

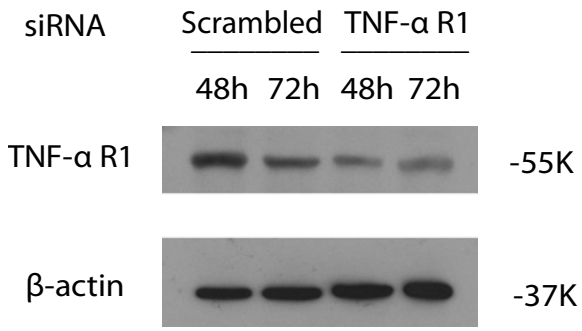

Figure 4.7 TNF-α receptor 1 is knocked down by siRNA in HLF-1 cells.
HLF-1 cells were transfected with 100 nmol scrambled or TNF-α receptor 1 siRNA utilizing DaharmFect 4. After 48 or 72h transfection, HLF cells lysates were analyzed for TNF-α receptor1 by immunoblot analysis. This experiment was repeated 3 times.

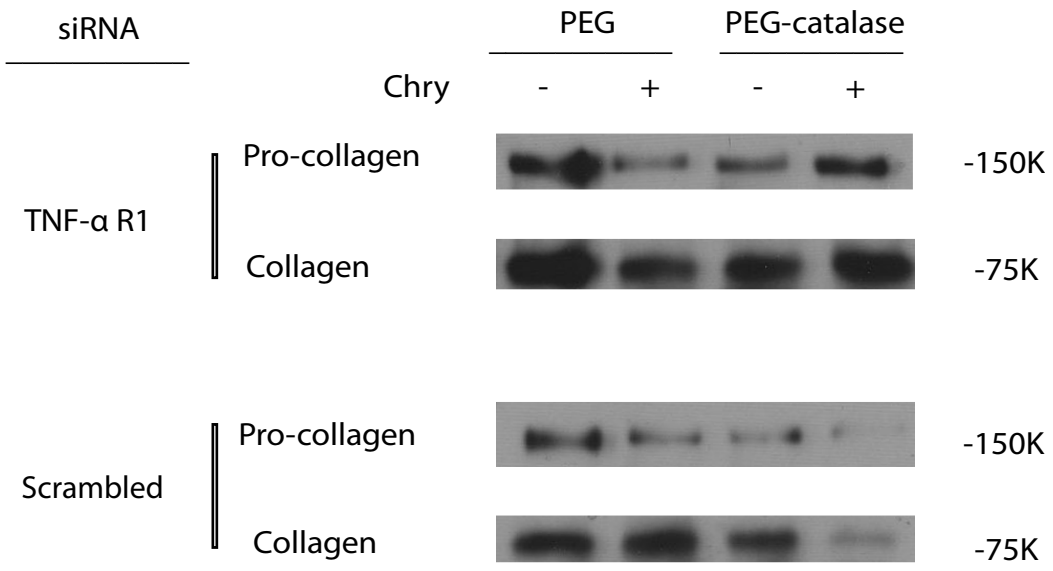

Figure 4.8 Collagen secretion is significantly greater in fibroblasts expressing TNF-α receptor 1.
HLF-1 cells were transfected with 100 nmol scrambled or TNF-α receptor 1 siRNA utilizing DaharmFect 4. After 72h transfection, medium was removed and HLF cells were cultured in conditioned media from THP-1 cells treated by PEG-catalase. After 24h, medium were collected and collagen expression was determined by immunoblot analysis.

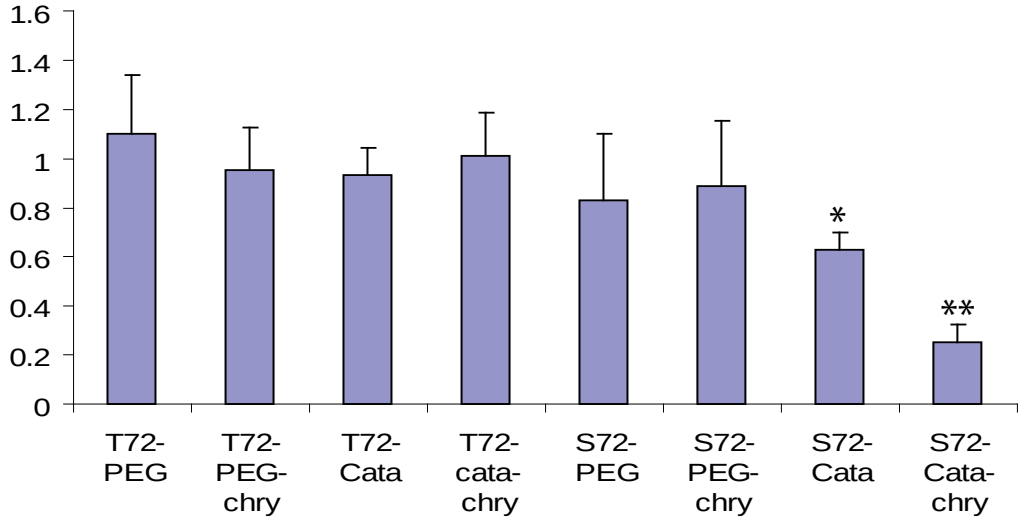

Figure 4.9 Densitometry of collagen in 3 experiments using TNF-α receptor 1 siRNA.

HLF-1 cells were transfected with 100 nmol scrambed or TNF-α receptor 1 siRNA utilizing DaharmFect 4. After 72h transfection, medium was removed and HLF cells were cultured in conditioned media from THP-1 cells treated by PEG-catalase. After 24h, medium were collected and analyzed for collagen by Western blot. Three separate experiments were analyzed by densitometry. n= 3, *, p<0.01, S72-Catalase versus T72 Catalase; S72-Catalase + chrysotile versus T72 Catalase + chrysotile. n= 3, **, p<0.05, S72-Catalase + chrysotile versus S72-Catalase.

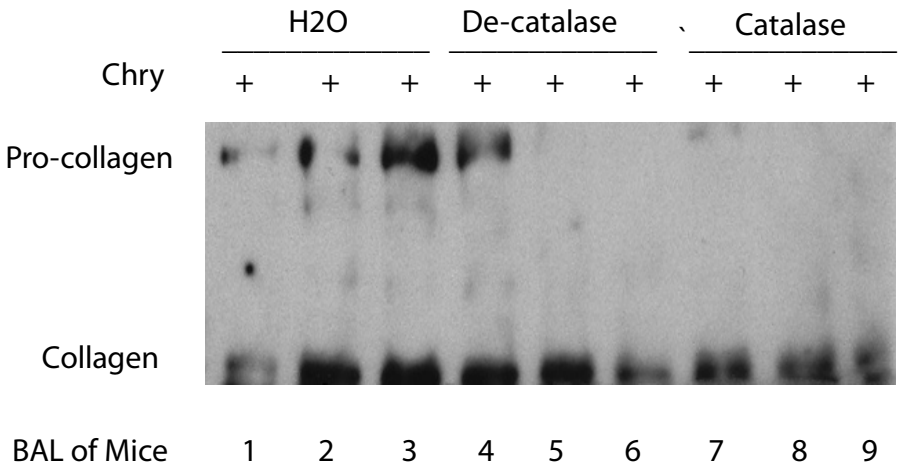

Figure 4.10 Catalase decreases collagen expression in BAL of mice exposed to asbestos.
WT C57BL/6 mice were intratracheally administered 100μg/mouse chrysotile asbestos in 50μl of normal saline. Then mice were divided into three groups, and daily administered with water, heat-denatured catalase and catalase (2,000 units/mouse), respectively for 20 days. On day 21, mice were euthanized and BAL fluid was collected for collagen expression.

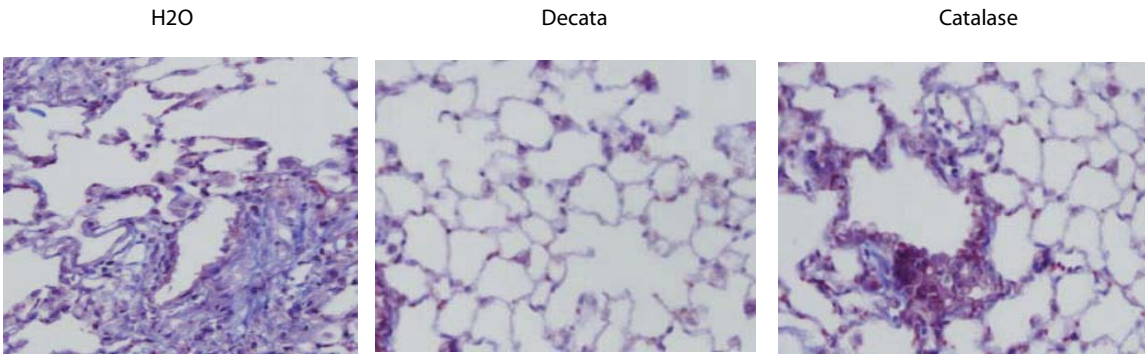

Figure 4.11 Fibrosis is attenuated in mice administered catalase.
WT C57BL/6 mice were intratracheally administered 100ug/mouse chrysotile asbestos in 50ul of normal saline. Then mice were divided into three groups, and daily administered with water, heat-denatured catalase and catalase (2,000units/mouse), respectively for 20 days. On day 21, mice were euthanized, and lungs were removed and processed for collagen deposition using Masson's trichome stain.

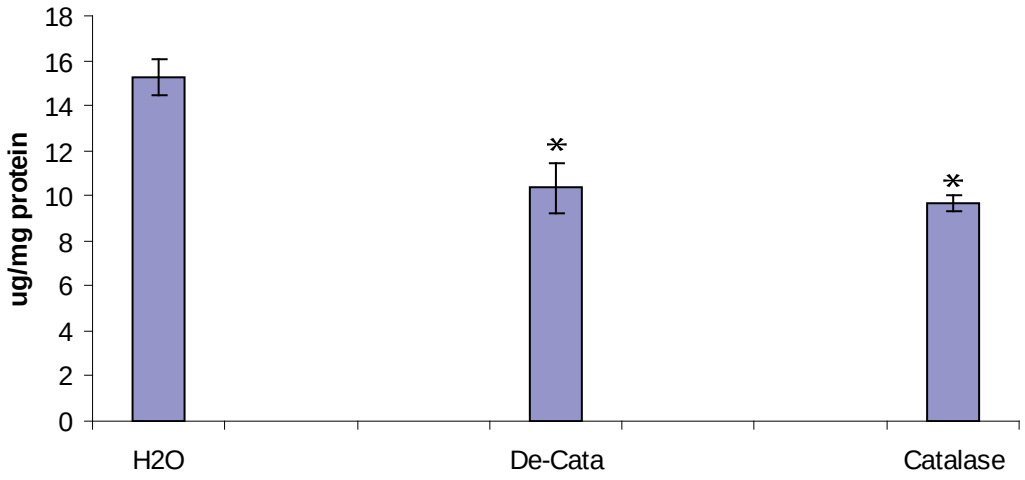

Figure 4.12 Hydroxyproline concentration is significantly decreased catalase-treated mice.

WT C57BL/6 mice were intratracheally administered 100μg/mouse chrysotile asbestos in 50μl of normal saline. Then mice were divided into three groups, and daily administered with water, heat-denatured catalase and catalase (2,000units/mouse), respectively for 20 days. On day 21, mice were euthanized and hydroxyproline concentrations in tissues were determined. n= 3, *, p<0.05, catalase treatment or denatured catalase treatment versus water control.

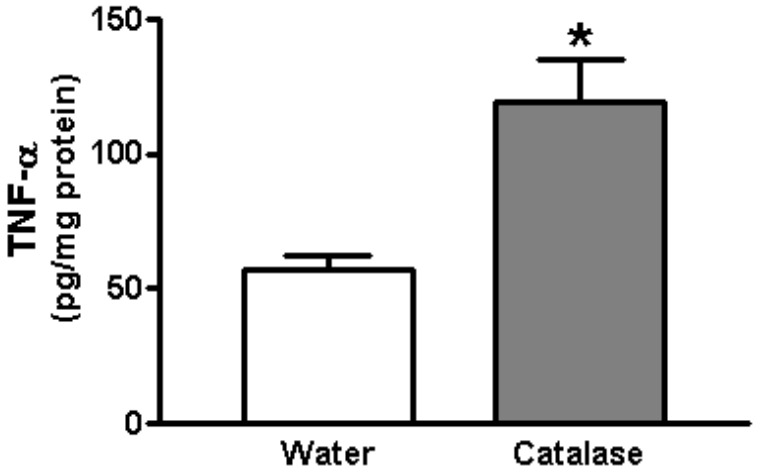

Figure 4.13 TNF-a is increased in BAL fluid obtained from mice exposed to catalase. WT C57BL/6 mice were intratracheally administered 100μg/mouse chrysotile asbestos in 50μl of normal saline. Then mice were divided into three groups, and daily administered with water, heat-denatured catalase and catalase (2,000units/mouse), respectively for 20 days. On day 21, mice were euthanized and BAL supernatants were collected for ELISA analysis of TNF-α. n= 3, *, p<0.05, catalase treatment versus water control.

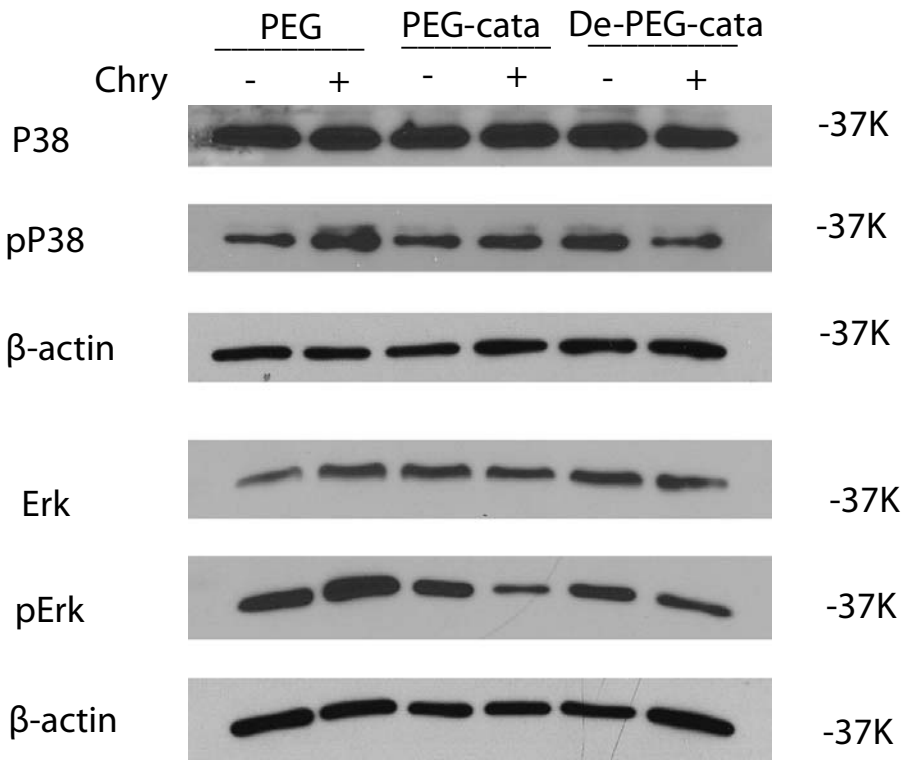

Figure 4.14 Conditioned medium of THP-1 treated by PEG-catalase analyzed for P38 and ERK kinases by Western blot.
THP-1 cells were treated with PEQ-catalase (100 units/ml) or heat denatured (95°C, 30 min) PEQ-catalase for 24h. For the last 4h, cells were exposed to chrysotile asbestos (10μg/cm^2). Conditioned medium was analyzed for P38 and ERK, phosphorylated P38 and ERK by Western blot. This experiment was repeated 3 times.

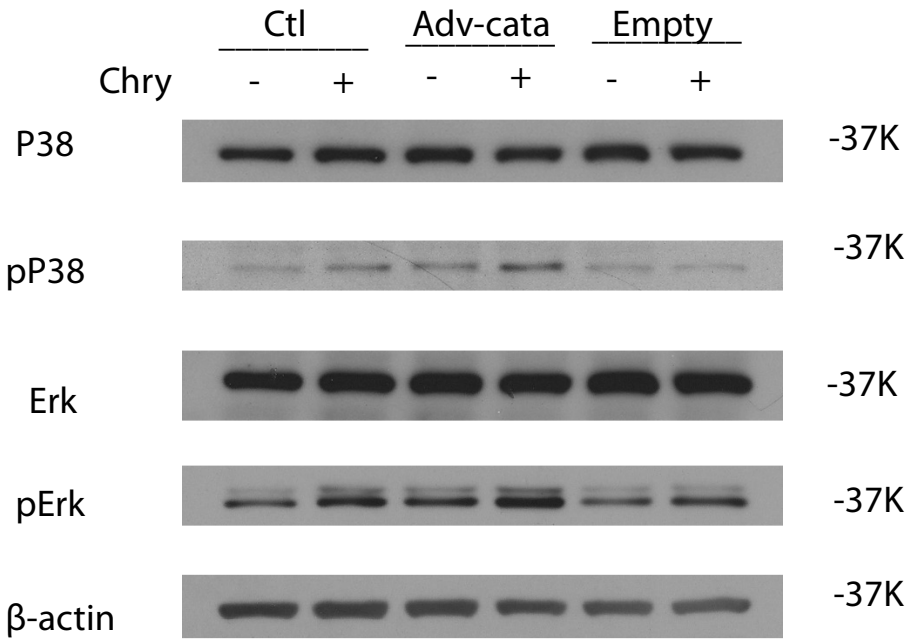

Fig 4.15 Conditioned medium of THP-1 treated by Adv-catalase analyzed for P38 and ERK kinase by Western blot.

THP-1 cells were transfected with catalase-expressing Adv-vector (500moi) or empty vector in serum free medium for 6h. Add to 0.1% serum for another 42h. For the last 4h, cells were exposed to chrysotile asbestos (10μg/cm^2). Conditional medium was analyzed for p38 and ERK, phosphated p38 and ERK by Western blot. This experiment was repeated 3 times.

CHAPTER 5

DISCUSSION

The major finding of our study is that catalase attenuates asbestos-induced pulmonary fibrosis while increasing pro-inflammatory cytokines. These observations are novel in that they contrast the prior dogma that pro-inflammatory states induce fibrosis development. These observations also uncover a therapeutic target to attenuate or halt the progression of pulmonary fibrosis.

In response to asbestos exposure, alveolar macrophages and recruited monocytes release ROS, including H_2O_2, and pro-inflammatory cytokines. Pro-inflammatory cytokines have been show to have both a positive and negative effect on fibroblast collagen production (Kalluri et al., 2006). However, excessive collagen deposition occurs when fibroblasts differentiate into myofibroblasts, which typically results in the development of fibrosis. Our observations suggest that pro-inflammatory cytokines abrogate collagen production.

H_2O_2 is generated by asbestos exposure in multiple cell types. Our studies have shown that mitochondrial H_2O_2 generation in macrophages exposed to asbestos is linked to the fibrosis development because inhibition of mitochondrial oxidative stress attenuates pulmonary fibrosis (Murthy et al., 2010; He et al., 2011; Osborn-Heaford et al., 2012). The primary function of catalase is converting H_2O_2 into water and oxygen. The enzymatic function of catalase has been shown to regulate cytokine release, kinase activation, and cell apoptosis (Goyal and Basak, 2010). Although studies have shown that catalase can attenuate fibrosis induced by asbestos (Murthy et al., 2009; Mossman et al., 1989), its role in mediating pro-inflammatory gene expression is not known

We have found that catalase decreases collagen production *in vitro* and pulmonary fibrosis *in vivo* despite increasing pro-inflammatory cytokine gene expression. TNF-α is a prototypical pro-inflammatory cytokine. The effect of TNF-α on fibroblast collagen production was investigated because our prior studies demonstrate that mice protected from developing asbestos-induced fibrosis had higher levels of TNF-α in BAL fluid (He et al., 2011; Osborn-Heaford et al., 2012). Fibroblasts cultured in BAL fluid from catalase-treated mice or conditioned media from macrophages exposed to PEG-catalase had decreased collagen production. These observations suggest that TNF-α may directly alter the ability of the fibroblast to generate collagen.

The mechanism(s) by which fibroblast collagen production was regulated includes apoptosis, necrosis, and senescence. To determine if TNF-α had a direct effect on fibroblast function, we knocked down TNF-α receptor-1 in fibroblasts because this receptor is associated with apoptosis. In response to asbestos, the conditioned media of monocytes treated with catalase induced less collagen production in fibroblasts, while collagen production was significantly increased in cells with TNF-α receptor-1 knock down. *In vivo*, mice administered catalase showed decreased collagen levels in BAL, and had significantly less hydroxyproline in the lung compared to mice that received the vehicle. In future experiments it may be useful to evaluate TNF-α receptor-1 function in fibroblasts by immunoprecipitation of TNF-α from conditioned media (or BAL fluid) or knock down TNF-α gene expression in macrophages. Rowlands et al. (2011) reported that endothelial mitochondrial Ca^{2+} regulates TNF-α receptor 1 shedding and thereby determines the severity of soluble TNF-α-induced microvascular inflammation. Our results show that TNF-α has an important function in regulating collagen production;

however, we did not evaluate shedding of the receptor or the role of mitochondrial Ca^{2+} regulated TNF-α receptor-1. Taken together, these data suggest that pro-inflammatory cytokine gene expression is inversely related to H_2O_2 levels and may play an important role in regulating fibroblast collagen production.

Based on the *in vitro* data showing increased TNF-α gene expression in asbestos-exposed macrophages treated with PEG-catalase, we investigated whether this would also be seen in a murine model of fibrosis. We have shown that catalase administration attenuates fibrosis development in mice exposed to asbestos, but we also found that intratracheal catalase administration increased TNF-α levels in BAL fluid compared to the mice that received the vehicle (water), which developed fibrosis. The cell count in the BAL was similar in both groups (data not shown). In aggregate, these data suggest that catalase, in a similar fashion as *in vitro*, attenuates fibrosis while increasing pro-inflammatory cytokines *in vivo*.

In comparison with PEG-catalase, "heated-denatured" catalase, which had 8% of the activity PEG-catalase, also increased TNF-α and IL-1β gene expression in macrophage suggesting that pro-inflammatory cytokine production is sensitive to slight changes in oxidative stress. In addition, "heat-denatured" catalase also decreased collagen production in fibroblasts. Kirkman et al. (1984) suggested that catalase might function as regulatory protein, releasing NADP+ to activate glutathione peroxidase to degrade H_2O_2 in erythrocytes with oxidative stress. According to Gaetani et al. (1996), glutathione peroxidase/reductase mechanism did not occur until more than 98% of the catalase had been inactivated. The heat-denatured catalase used in our studies was 92% inactive, so it is unlikely that glutathione peroxidase function increased to alter H_2O_2

levels. Future experiments to completely inactivate catalase enzymatic activity with azide or cyanide will be necessary to further investigate the role of catalase in pulmonary fibrosis.

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