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The Cell Regulatory Systems affected by Asbestos induced Mesothelial Cell Autoantibodies

by

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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Robert Hanson find it satisfactory and recommend that it be accepted.

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List of Abbreviations

- AFA: Antifibroblast bodies
- CARD: Center for Asbestos Related Disease
- ECM: Extracellular Matrix
- EGFR: Epethelial Growth Factor Receptor
- FPLC: Fast Protein Liquid Chromatography
- IgG: Immunoglobulin G
- LA: Libby Amphibole
- LPT: Lamellar Pleural Thickening
- MMP: Matrix Metallo Protease
- MS: Mass Spectrometry
- Met5a: Mesothelial Cell line 5a
- MCAA: Mesothelial Cell Autoantibodies
- OD: Optical density
- PDGFR: Platelet Derived Growth Factor Receptor
- PLG: Plasminogen
- PM: Plasma Membrane
- ROS: Reactive Oxygen Species
- TGF: Transforming Growth Factor
- TGFB: Transforming Growth Factor Beta
- TBS: Tris buffered saline
- TRK: Tyrosine Receptor Kinase

Abstract

Lamellar Pleural Thickening is a fibrotic disease induced by exposure to Libby Amphibole (LA) asbestos that causes widespread scarring around the lung, resulting in progressive loss of pulmonary function. In the effort to understand the pathogenesis of this disease, it has been shown *in vitro* that mesothelial cells (Met5a) exposed to MCAA increase collagen deposition into the Extracellular Matrix (ECM). In this study, we sought to further elucidate why the presence of MCAA would result in increased collagen deposition by looking to see if phosphorylation pathways were upregulated. We also identified the protein targets bound by MCAA on the cellular surface using biotinylation to label and isolate surface proteins. We were able to show that there are multiple proteins being targeted by MCAA that could contribute to excessive collagen deposition. We were also able to show strong evidence for investigating the contribution of plasminogen to the development of this disease.

Chapter 1

Introduction

Background

This study is part of a larger effort to elucidate the mechanism by which progressive Lamellar Pleural Thickening (LPT), an emerging fibrotic disease found in Libby Montana, develops. To give context behind the methods employed in this section of the research, we will discuss the role of autoimmunity in fibrosis. The evidence that amphibole asbestos exposure causes autoimmunity, and the preliminary work that led us to the results presented in Chapters 2 and 3.

Fibrosis and Autoimmunity

Fibrosis is a general term that describes an abnormal accumulation of fibrous tissue within the body. In specific cases, such as pleural fibrosis, this accumulation of excess tissue can be fatal. The bulk of this extra tissue is composed of extracellular matrix components, such as collagen. Most of these substances play a key role in the wound healing process. The body's cell signaling pathways that facilitate healing and maintaining healthy tissue are tightly controlled. It is believed that fibrosis is the result of the malfunction of these processes.

While there are fibrotic diseases that can be accounted for as a result of exposure to toxic substances or repetitive injuries, there is a large volume of work that indicates the presence of autoantibodies could contribute significantly to the development of abnormalities observed in some forms of fibrosis, such as Systemic Lupus or Scleroderma [1–3].

One widely used model states that autoantibodies are generated as the result of the adaptive immune system being presented with portions of the body's cells that were not properly degraded through apoptosis. This is assumed to happen when a large amount of cell death occurs in a short amount of time. A key marker of such events is a large concentration of reactive oxygen species (ROS)[4]. In such an event, antibodies that target self-proteins are generated. Generally, such autoantibodies lead to immune-mediated target cell damage.

Instead of triggering attacks from the immune system, it has been hypothesized that these autoantibodies are dysregulating mechanisms of ECM homeostasis by affecting cell signaling processes. Since a cell signaling process can have upwards of 20 proteins that influence its outcome, researchers often use Top Down theoretical models to design and carry out their experiments related to the development of fibrosis.

In the efforts to understand the effects of autoimmunity on fibrosis, researchers have focused on the cells that are responsible for maintaining the extracellular matrix. Two key cells of interest have been fibroblasts and mesothelial cells[5,6]. It was demonstrated by Baroni that collagen type 1 gene expression in fibroblasts increased in response to stimulatory autoantibodies binding to the tyrosine kinase receptor PDGFR [7]. This is significant because type I collagen has been shown to be a major component of fibrotic tissue[8,9].

While many signaling mechanisms exist which could account for increased collagen expression, it has been demonstrated that cell-signaling

pathways that involve phosphorylation can affect the outcomes present in experimentally induced versions of fibrosis. Using proteomics and an analog of the fibrosis inhibitor mannose-6-phosphate, Schitler demonstrated that the TGF signaling pathway, a kinase mediated pathway, was down-regulated as well as collagen production [10]. Using a murine model and various kinase inhibitors, Les Cras was able to prevent further development of fibrosis in experimentally induced versions of fibrosis. [11,12]. These and other data have proven invaluable to those researching emerging autoimmune diseases like Lamellar Pleural Thickening as it is seen in Libby Montana.

A Brief History of Asbestos and Lamellar Pleural Thickening

Asbestos is a term used to describe six substances that have been used commercially for decades and possess a similar mineral habit or shape. The following substances are classified as asbestos: Chrysotile, Crocidolite, Amosite, Tremolite, Anthophyllite, and Actinolite. However, many other mineral fibers have very similar properties, including severe health effects, and are classified as amphiboles even though they are not typically used for commercial purposes (Meeker, Metcalf). Each one of these substances is a silicate chain containing some additional metal ions incorporated into the basic formula. The feature common to all forms of asbestos is that the individual fibers have an elongated fibrous shape, with an aspect ratio greater than 3:1 [15]. These fibers can be found in bundles that can be broken or cleaved into smaller fibers through mechanical means[13]. Of these substances only Chrysotile is considered to be serpentine, making it drastically different at the microscopic level from the other types of

known asbestos. The other forms are all given the sub-classification of amphibole asbestos. These forms of asbestos can be identified by the straight spikey shape of their individual fibers [13]. The dangers associated with asbestos exposure have led to discontinuance of mining operations in the U.S.A. However, naturally occurring asbestos has been found in many areas of the United States that are being disturbed by public or corporate use. Exposures have been reported from the disturbance of natural features in California, Nevada, and Montana [14,15]. In addition to this, asbestos was mined and used heavily in the U.S. from the early 1800's until the government began the slow process of bringing it under regulation as part of the toxic substance control act in 1976. With nearly 200 years of putting asbestos into buildings and other public facilities, it's clear that avoiding asbestos exposure entirely during one's lifetime is very unlikely.

There are multiple asbestos related diseases that have been classified. Our study centers around Lamellar Pleural Thickening (LPT) as it is seen in Libby Montana, where extensive asbestos exposure occurred due to the mining of amphibole-contaminated Vermiculite. This is important to indicate because LPT results in distinctly different symptoms compared to other asbestos related diseases[16].

From the clinical perspective, LPT causes a diffuse thickening of the pleural lining surrounding the lung to occur in a progressive fashion. Eighteen percent of the 6,668 people screened in Libby in 2000-2001 were found to have pleural abnormalities. More recently, rates of pleural disease in Libby have been reported to be as high as 50% (Antao 2012). In this group, loss of pulmonary

function was accelerated in comparison to other similarly exposed groups[16,17]. Noonan showed that exposure to Libby Amphibole (LA) asbestos is correlated with an increased likelihood of developing autoimmune diseases [18], and our lab showed that the presence of serum autoantibodies was associated with lung disease in the Libby population (Pfau 2005). This raised the possibility that an autoimmune process was contributing to this progressive lung disease.

In addition to autoantibodies that affected fibroblast growth, our group's studies have shown that asbestos autoantibodies were generated that targeted mesothelial cells[19]. Termed Mesothelial Cell Autoantibodies (MCAA), these autoantibodies were of particular interest because of the potential role that mesothelial cells play in the pathogenicity of fibrosis. An excellent review by Mutsaers details some additional information about mesothelial cells[6]. Using serum containing these MCAA, Serve was able to show that collagen deposition was upregulated *in vitro*. Serve also showed that when IgG was removed from MCAA positive serum, the upregulation in collagen deposition was not observed [20]. These results brought the research group's efforts to the question this study attempted to answer: What is the identity of the protein or proteins targeted by MCAA? To answer this question, we turned to the constantly improving technology of mass spectrometry based proteomics.

Proteomics Utilizing Tandem Mass Spectrometry

Statistical analysis of the proteome, also known as proteomics, has led to a rapid acceleration in the pace of biological research. The instrument that has

facilitated much of this growth is the Mass Spectrometer (MS). By using magnetic fields to manipulate the path-length an ionized molecule must take reach a detector, this instrument is able to accurately discern the mass of different components in a molecule. This is expressed in terms that relate the mass of the molecule to the charge it received during the ionization process (m/z).

Mass spectrometry has supplemented many of the previous methods as an initial step for hypothesis generation and is often used in elucidating the differences between control and disease states. This portion of the review discusses the general types of discovery proteomics, mass spectrometry experiments, and the methods used to prepare plasma proteins for MS/MS analysis. In general, there are two basic workflows for analyzing a protein via mass spectrometry, Top Down and Bottom Up. Top Down workflows are designed to work with complete intact, linearized proteins. In contrast, Bottom Up workflows work with a solution of proteins that have been broken down into peptide fragments. Both designs have advantages and weaknesses.

Top Down workflows provide great utility in regards to identifying proteins because the entire protein can simply be analyzed for its sequence in a single analysis. This is a clear advantage compared to Bottom Up workflows, where the protein is always cleaved into peptide fragments before analysis. The consequence of this is that different portions of the peptide could potentially be analyzed out of sequence. Despite this obvious advantage, the challenges associated with keeping a complete protein in solution while injecting it into an

MS instrument are great. Also, because proteins are significantly larger and more complex than any other molecule analyzed with mass spectrometry methods, nonstandard equipment is required for a MS to analyze a complete protein. Specifically, a MS with Fourier transform ion cyclotron resonance capabilities is needed to obtain a signal to noise ratio that is low enough to discern mass differences accurately [21]. Unfortunately, the challenges mentioned here have limited the number of situations where Top Down Proteomics can be applied and will continue to do so until improvements are made.

Though improvements are constantly being made to Top Down methodology; developing statistical tools that interpret the complicated spectra associated with Bottom Up workflows has currently occurred at a more rapid pace. Because they do not need the specialized instrumentation required by Top Down proteomics, Bottom Up workflows have become the more common format of studies performed via MS/MS. In this workflow, an enzyme is used to cleave the reduced protein into peptide fragments small enough for the ionization process. The most common enzyme used is Trypsin because of its predictable fragmentation pattern. One technique used to increase confidence and coverage in the results obtained via MS/MS is to analyze identical samples that have been prepared with different proteolytic enzymes. This can be accomplished by separating a sample of interest into several aliquots and treating each aliquot with a different proteolytic enzyme. Each sample will then be analyzed via tandem MS. A high degree of confidence can be placed in any protein hits that

show up in the results of both samples because they were essentially obtained from two different profiles of peptide fragments.

When a sample is analyzed via tandem MS, it goes through two procedures that are linked together. The instrument first analyzes the sample for total number of fragments, identifying them by their distinct mass. The second procedure is then an iterative process. The spectrometer will cycle through each molecular mass detected and analyze the peptide sequence of the protein found at that specific mass. The resulting spectra contain a series of peaks that reflect different fragments of the peptide being analyzed. Irregularities resulting from the ionization process can give rise to erroneous peaks, but the occurrences of these are very low. Because the peaks are ordered from fragments of highest mass to those of lowest, the distance between these peaks can be measured to determine the primary sequence of the fragment.

Because mass spectrometry is often run on complex samples containing multiple proteins, data analysis is carried out by computers. The first step taken by computers is to analyze the spectra and remove any confounding peaks that have arisen from the sources described in the previous paragraph [22]. After this has been completed, the program generates all the reasonable possible sequences that could produce the observed spectra. Each possibility is then compared against the NCBI database through a BLAST search. If a homologous sequence is found, the sequence is assigned a score based on the strength of the BLAST search. The sequence with the highest score is assigned to that spectrum as the peptide match. If no matching data is found in the BLAST database, then

the sequence is labeled as no match. After all the spectra have been searched, the matches are ranked according to the number of spectra found for them. If two or more peptide fragments from the same protein are found in a sample, it is usually enough to confirm that protein's presence in the sample. This ability to identify proteins in samples with multiple components is a clear advantage possessed by tandem mass spectrometry when compared to other forms of peptide sequencing.

One major disadvantage to methods utilizing mass spectrometry is reproducibility. As complexity increases, the instrument's ability to provide clear data drops. Variability introduced in the preparation process is common enough that most researchers recommend having supporting data for information obtained using discovery experiments [23]. These are only a few of the reasons that it is desirable to provide protein used for Mass Spectrometric analysis in a pure, isolated form. Clearly, this raises some challenges in regards to applying proteomics to proteins located in the plasma membrane (PM) of cells.

Currently, plasma proteins make up a significant number of therapeutic targets[24,25] because they are located at the interface between the cell and its environment. Understanding their roles in regulating cellular processes is both vital to curing disease states and to avoiding unwanted side effects. The challenges that arise when studying plasma proteins stem from the properties that make them perfectly suited for completing their role within the cell.

Proteins located in the PM are problematic to isolate and prepare for MS because they contain both hydrophilic and hydrophobic domains. When released into solution, they tend to aggregate and precipitate out unless detergents are included [26]. Another feature that increases the difficulty of isolating plasma proteins is the wide variance in expression. A small number of proteins make up the majority of receptors that are constantly present. If the protein target of a pathogenic system is not one of these common receptors, it can be difficult to isolate enough protein to perform analysis. The task can be further complicated if the proteins must be left in their native state, which results in interactions that may prevent obtaining a completely pure sample.

The last decade has seen the development of many techniques that have increased the efficiency of isolating plasma proteins. Although these techniques cannot obtain all the proteins located in the membrane, they have greatly reduced the number of contaminating proteins, reducing the noise and increasing signal in the resulting spectra [27].

Until recently, the traditional method of isolating membrane bound proteins was gradient centrifugation. This method involves long processing times and only allowed a small fraction of the total plasma proteins to be isolated [26]. It involved homogenizing the cells into a lysate, performing a crude purification, followed by a more refined purification in sucrose gradient. Each step relied on the fact that heavy molecules settle faster than lighter ones, and would occupy a lower position in the gradient. Due to the poor yields of plasma

proteins from this method, many alternative methods have been developed in recent years.

One of the methods being refined to isolate the extracellular domains of plasma proteins is the practice of cell shaving. In this method, an enzyme is used in low concentration to shave off the extracellular domains of the cell. Cell shaving was suggested as an alternative method of plasma protein isolation for a variety of reasons. First, it removes the need to use detergents to solubilize the hydrophilic domains during preparation steps. Second, it avoids the complications introduced by cytosolic proteins being introduced into the sample [26,28]. The theory is sound but execution is problematic. Any surface proteins that lack a cut site at the appropriate location are likely to be excluded from the final suspension if it is performed properly. If it is performed improperly, cytosolic contamination can occur due to cell lysis, or no fragments may be liberated at all.

Some efforts to refine this process have been made. Cold adapted proteases were used to lower the occurrence of cell lysis [29]. Another research group used a lysate obtained from previously shaved cells in order to identify false positives in their experimental group [30]. Others have used an array of proteases in parallel to generate more complete profiles of the proteome present in the PM [31]. All these innovations have improved the results of mass spectrometry obtained from cells with robust membranes. But this technique still remains too blunt of an instrument to be effectively used for delicate cells.

Another method that is currently increasing in use is the practice of labeling proteins of interest. The end goal of this process is to use affinity chromatography to remove the labeled proteins from a lysate. Many different molecules have been used to achieve this end. Some of the more novel have included iron nanoparticles [32] and Silica colloidal beads[25]. Probably the most ubiquitous in its use is the biotin avidin method.

Biotin binds to avidin, or streptavidin, through multiple hydrogen bonds. Heat will disrupt these interactions, but as long as the reaction is left at room temperature the binding capacity of these columns is very strong. The process is versatile enough that products exist for labeling the C or N terminus of a peptide chain, as well labels that are lipid soluble. Some labels are fitted with a functional disulfide bond that can be reduced to release the proteins [25].These particular types of labeling molecules provide an extremely convenient method of elution from biotin columns and are sold under the commercial name of SS biotin.

The major limiting factors in this method are steric hindrance, a lack of an appropriate labeling site on the protein of interest, or poor solubilization of the plasma protein in question (Pierce Cell Surface Protein Isolation Kit, Life Technologies, Grand Island, NY). An interesting strategy may be to use cell shaving to remove the more ubiquitous surface proteins and then use a labeling reaction to obtain the remaining proteins. Another concern that was raised in this process is the composition of the functional group used to label the peptides. Weekes reported in a comparison study that protein isolated using SS biotin

contained intracellular protein. They suspected this could indicate that the SS biotin molecule is partially membrane soluble. They reported better results using biotin conjugated to an amino oxy functional group [25].

In many ways, these methods are still in development stages. Many of the papers published gauge the success on the number of proteins identified and whether the annotation matches those predicted[30,33]. The papers that utilize these methods assume that the quantities of expressed protein on the surface of a cell will grant the insight needed to implicate the causes of disease states. In many cases, they will be right. However, few of the preparation and isolation methods take precautions to maintain the native state of the proteins as they seek to increase the yield of protein obtained from the plasma membrane.

As an endpoint, most therapies are looking to manipulate biological systems by targeting a receptor that will need to be bound by a small molecule or protein. Therefore, the preparation methods fall short of the mark for identifying targets of autoantibodies and other disease causing agents that are similar in nature. This is why proteomics data is often published with supporting data obtained from other methods. This could very well change as enrichment strategies that respect the need to maintain native conditions are developed and proteomic methods increase their relevance in clarifying mechanisms of pathogenicity. Of the alternative methods currently available, only labeling techniques seem to take this need of maintaining the native state into consideration.

As was mentioned earlier, due to the variable nature of data obtained from discovery proteomics methodology, it is good to provide supporting information for another methodology to validate the information found. Many researchers find that using networking programs such as Cytoscape can expedite the process of finding support from the literature to validate information discovered in a proteomics experiment.

Bioinformatics

With the staggering volumes of information that are being generated about cellular processes in the many subset disciplines of biology, it has become nearly impossible for researchers to cover the breadth of advances being made while searching the literature by hand. To meet these challenges, computer scientists have been designing systems that connect and compile related data. Pathway networks combined with web annotation have become invaluable tools to aid researchers in the process of searching the literature. In fact, networking programs have become so prevalent that programs have been created to help researchers integrate the networking tools pertinent to their project in one location. The most valuable consequence of these databases is that it has allowed many research groups to take a multi-disciplinary approach to answer key biological questions in the pathogenesis.

Aims

The overall goal of this study was to identify the cell surface proteins targeted by MCAA, which would contribute to excessive collagen deposition

when bound. The approaches utilized to answer this question expanded as initial approaches produced less than satisfactory data. Our aims have been modified to reflect this change.

Aim 1: Isolate the protein bound by MCAA and identify it using Bottom Up Tandem Mass Spectrometry and proteomic analysis.

Aim 2: Investigate Thy-1 and other general cell signaling pathways for involvement in the increase in collagen deposition.

Aim 3: Utilize pathway networking programs such as Cytoscape to direct experimentation and confirm the results of experiments.

The next section will discuss the steps that were taken to optimize the procedures used to achieve these aims.

Optimization of Methods

Aim 1: Because cell signaling within a cell involves multiple interactions, a large variety of techniques can be employed to look at the state of the cell and derive the specific signaling pathways that are being affected by the binding of MCAA to their cellular target(s). Of course, the most direct way to begin the process of elucidating the mechanism of pathogenesis would be to identify the targets being bound by MCAA. To do this we needed to employ techniques that involved protein isolation and identification followed by bioinformatics analysis. In theory, this would identify a surface protein, most likely a receptor. This would provide a good starting point for confirming the cell signaling pathways that are responsible for the excess collagen deposition observed by Serve[20].

One of the first tasks in determining the exact identity of an antigen is to remove all the other proteins not being bound by MCAA. The first strategy used was to separate the surface proteins by size on SDS-PAGE and then probe using a Western blot technique. However, no bands were visible on the Western blot probed with serum MCAA (Figure 1.1). To double check, we performed a dot blot which showed binding to the native proteins simply dotted on the PVDF membrane, then probed with the serum MCAA. Based on the strongly stained protein in the dot blot, we deduced that the epitope's structure was dependent on the 3D structure of the protein, which would require a native gel for visualization. A native Western has been shown to maintain the 3D structure of a protein. However, our findings using the native Western approach showed that we were unable to demonstrate any observable binding (data not shown).

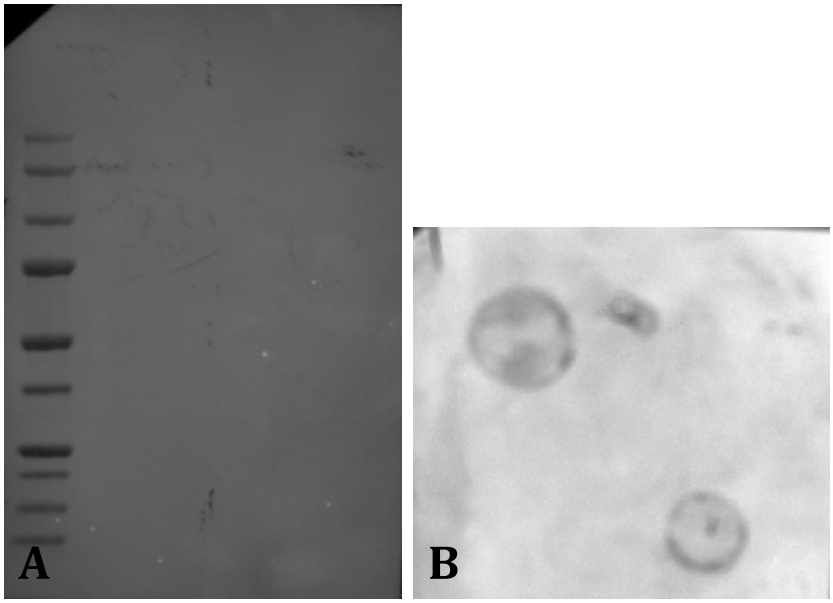

Figure 1.1. Representative result of our Western blot and Dot blot procedure: A) A Western blot that shows that while the protein ladder was clearly transferred, no other bands in the experimental lanes displayed any binding. B) A dot blot shows binding at both 10 uL and 20 uL of concentrated lysate. Control Blots (not shown) were checked with Ponceau S. to ensure that protein was present.

The next technique used was immunoprecipitation to extract the MCAA-bound target antigen(s) out of a protein lysate. However, the results were less than satisfactory when the bound proteins were eluted and run out on a gel. We had each band from the gel analyzed via MS, but most of the bands contained little more than IgG or cytoskeletal keratin. The searches we did on the unique results showed that we had not succeeded in isolating any surface proteins that were linked with the upregulation of type 1 Collagen.

The next technique we opted to use was cell shaving, in order to retrieve the peptides of surface proteins still in their folded conformation. This utilizes an enzyme with a site specific cut site, such as trypsin, to shave off the extracellular domain of the proteins from the cells [30]. These proteins may then be concentrated and separated according to size using size exclusion chromatography. This technique uses a column packed with gel beads that are covered with micropores all over their surface to separate proteins according to their various molecular weights. The peptide strands that are small will fit into the micropores and as a consequence, will travel through the column at a slower rate than the proteins of larger molecular weight.

It is important to remember that, because the proteins have been left in their native state, they may still interact with other proteins while being separated. This will result in samples that are still a mixture of proteins. However, size exclusion can still partially separate the proteins recovered from the cell surface lysate thus reducing the overall complexity of the samples we tried to analyze via other methods. We then took the protein fractions derived

by fast protein liquid chromatography (FPLC) and tested them via ELISA for binding by the MCAA (Figure 1.2).

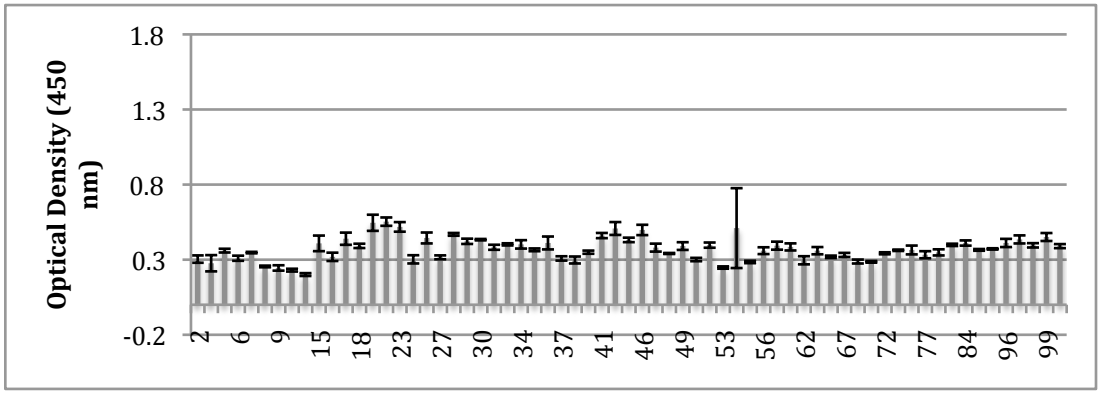

Figure 1.2. ELISA for MCAA binding of cell shaving lysate separated by MW: The lysate was separated using a size exclusion column. 200 ng of each fraction was added to a well in a 96 well plate. Wells were stained using a protein lysate ELISA protocol.

This size exclusion procedure was also performed on lysate that had been prepared using biotinylation to label the surface proteins and then isolate them via affinity chromatography. The results of this method are incorporated into Chapter 2.

Aim 2: The purpose of Aim 2 was to test potential receptors that had already been identified in the literature. The first such receptor we tested was Thy-1, a GPI-anchored surface protein that has been shown to regulate myofibroblast activity. Thy-1 positive fibroblasts are quiescent and do not produce collagen, suggesting that Thy-1 is an intercellular signal that maintains an anti-fibrotic state [34]. However, Thy-1 negative fibroblasts are activated to become myofibroblasts that are pro-fibrotic. Early work in the Pfau Lab had shown that asbestos treatment led to the release of Thy-1 from cultured fibroblasts. The hypothesis was that, once exposed to free Thy-1, the immune

system might produce autoantibodies that would target cell surface Thy-1 and inactivate it, leading to increased, and more chronic, fibrosis. The first test we performed was for the presence of Thy-1 on our fibroblast cells. This was done using a flow cytometry protocol in which the Thy-1 receptor was bound with a primary antibody and a general secondary antibody conjugated to an AlexaFluor 488 molecule. Differential intensity was seen between the background (secondary antibody only) staining and those stained with anti-Thy-1, demonstrating the presence of surface Thy-1 (Figure 1.3).

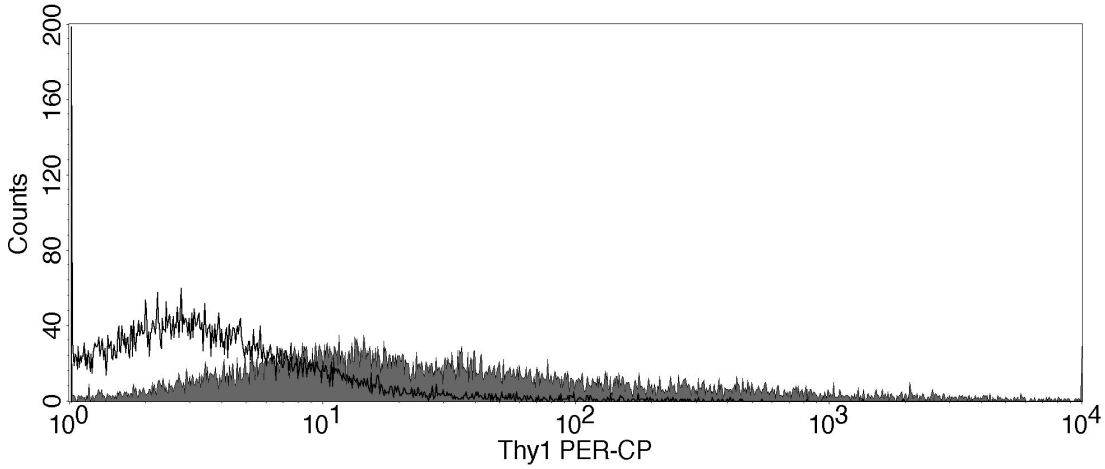

Figure 1.3. Presence of Thy-1 on L929 fibroblasts: L929 cells that had been treated with a goat AlexFlour488 anti mouse secondary to test for nonspecific binding (clear overlay) were compared to L929 cells that had been stained with a anti-Thy1 primary and anti mouse secondary (dark gray).

To test for binding of MCAA to Thy-1, we employed a competitive assay in which we exposed L929 fibroblasts to MCAA+ serum and anti-Thy-1 to see if they would compete for the same binding site. As can be seen in Figure 1.4, the commercial antibody did not inhibit the MCAA binding.

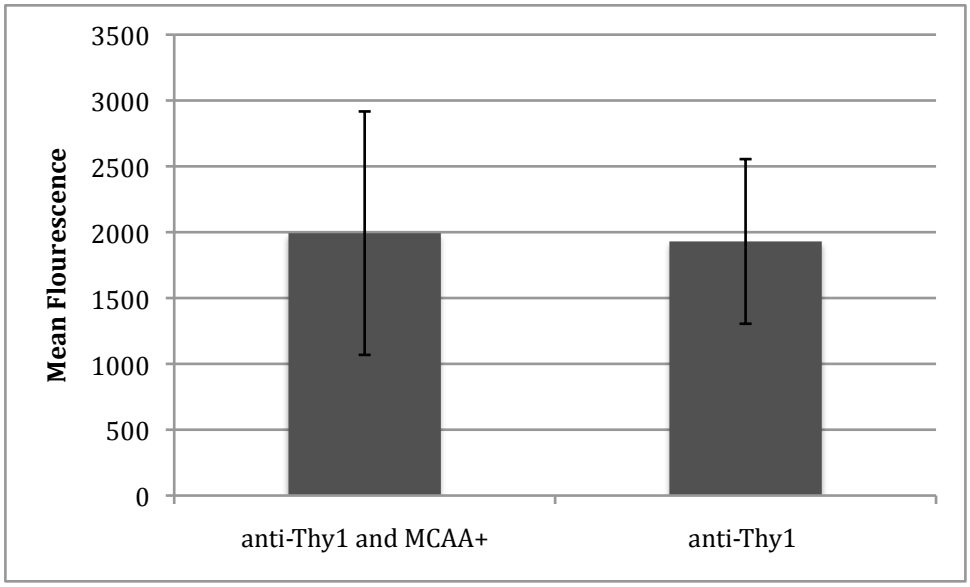

Figure 1.4. Competitive binding assay between antithy-1 and MCAA: MCAA was added to fibroblasts prior to the addition of anti-Thy1 antibodies to see if MCAA would compete for the same binding site on Thy-1.

Unfortunately, a negative result in this test leads to no clear conclusion, as all we showed is that the autoantibodies did not compete for the same binding site as our commercial Thy-1 antibody. After this result and a few other attempts failed to generate useful insight, we stepped back and reexamined our approach. At this point we decided to test cell signaling using a broader technique before zeroing in on more specific pathways through phosphorylation. Those techniques and data are included in Chapter 3.

Aim 3: To probe online gene databases, we selected Cytoscape version 3.0.2 . After surveying the plugin programs available, GeneMania was selected to use for generating the network. Type 1 collagen was chosen as a starting point for network generation because it has been shown to undergo dramatic changes in the development of pulmonary fibrosis [8]. A network of 21 genes connected to COL1A1, the gene responsible for generation of Type 1 collagen, was generated.

From there, a network of 200 genes connected to those genes was created. This process was repeated until a network of 2000 nodes had been assembled. Search terms were entered to probe the annotation. Some examples of these terms were: plasma membrane, collagen, surface protein, and antigen. Any genes that lit up were searched on Google Scholar for connection with fibrosis. Without a priori knowledge, it was difficult to select terms that would highlight meaningful connections, so the project was mostly shelved for six months.

Upon receiving initial data from our collaborators in Moscow, the networks were probed again using information from the mass spectrometry data to determine the search terms. An additional protein of interest from the mass spectrometry data was found this way. The mass spectrometry data was also compared with a protein interaction network titled STRING. This also yielded positive connections that were weighted by the strength of the literature supporting their displayed connections. These data are incorporated into Chapter 2.

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Chapter 2

Libby Amphibole-Induced Mesothelial Cell
Autoantibodies Bind to Surface Plasminogen and Alter Collagen Matrix Remodeling

Libby Amphibole-Induced Mesothelial Cell
Autoantibodies Bind to Surface Plasminogen and Alter Collagen Matrix Remodeling

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Abstract

Lamellar Pleural Thickening (LPT) is a fibrotic disease induced by exposure to Libby Amphibole (LA) asbestos that causes widespread scarring around the lung, resulting in progressive loss of pulmonary function. Investigating the effects of autoantibodies to mesothelial cells (MCAA) present in the study populations has been a major part of the effort to understand the mechanism of pathogenesis. It has been shown *in vitro* that mesothelial cells (Met5a) exposed to MCAA increase collagen deposition into the Extracellular Matrix (ECM). In this study, we sought to further elucidate why the presence of MCAA would result in increased collagen deposition by identifying the protein targets bound by MCAA on the cellular surface using biotinylation to label and

isolate surface proteins. Protein targets were selected by immunoprecipitation and MCAA binding via ELISA. The fractions that demonstrated binding by MCAA were then analyzed by tandem mass spectrometry and MASCOT analysis. We identified annexin A5, cytoskeletal keratin 18, and plasminogen as possible candidate targets that could affect the regulation of the ECM. The most promising result from the MASCOT analysis, plasminogen, was tested for MCAA binding using purified human plasminogen in an ELISA. We report that serum containing MCAA bound at an optical density (OD) 3 times greater than that of controls, and LA-exposed subjects had a high frequency of positive tests for anti-plasminogen autoantibodies. This work implicates the involvement of the plasminogen/plasmin system in the mechanism of excess collagen deposition in Met5a cells exposed to MCAA. Elucidating this mechanism could contribute to the understanding of LPT.

Introduction

Lamellar Pleural Thickening (LPT) is an emerging disease distinguished from other forms of pleural fibrosis because it results in a diffuse thickening of the pleural lining coupled with a progressive loss of pulmonary function [1]. Various forms of pleural fibrosis occur in individuals who suffer from pleural infections or other damage to the mesothelial lining, such as exposure to asbestos. However, LPT has so far only been described among individuals exposed to Libby Amphibole (LA) asbestos [1,2]. In the amphibole-exposed population of Libby MT, pleural fibrosis is the predominant cause of morbidity and mortality [3,4], and it, like other forms of pleural fibrosis, is refractory to

treatment, leaving patients and physicians without many options to prevent respiratory decline. In view of the discovery of extensive exposures to similar amphiboles in the southwestern U.S. [5,6], many new cases of LPT may appear, necessitating new treatment modalities.

Previous studies have shown strong correlations between the presence of mesothelial cell autoantibodies (MCAA) and the existence of conditions that could lead to the development of LPT [7,8]. Specifically, among Libby subjects, the presence of MCAA in serum was associated specifically with pleural, but not interstitial, disease[7]. *In vitro*, exposure to MCAA increased collagen matrix formation by cultured human mesothelial cells [8]. In order to further the work of elucidating a mechanism of pathogenesis, it is necessary to identify the cell signaling events that are being triggered as a result of MCAA binding to its cellular target.

Tandem mass spectrometry can be used for both exploratory and hypothesis directed proteomics. This is due largely to the bioinformatics tools available to analyze the peptide sequences identified by the method[9,10]. In the case of a pure protein sample, it may provide positive identification. In the current study, our hypothesis was based on previous work identifying platelet-derived growth factor receptor alpha (PDGFR-α) as a potential target for anti-fibroblast antibodies (AFA) in asbestos-exposed mice and in patients with scleroderma [11,12]. In both cases, the autoantibodies stimulated cultured fibroblasts to produce collagen, making PDGFR-α a potential target for the MCAA as well. The exploratory component of our study was necessitated by the lack of

any previous work regarding stimulatory antibodies to mesothelial cells and the presence of multiple potential pathways that could impact collagen matrix formation.

In this study we sought to identify the antigen(s) bound by MCAA on the surface of human mesothelial cells. We proposed to accomplish this through biotinylation of the surface proteins, followed by analysis via tandem mass spectrometry. We followed up on the most promising candidate by testing for binding by MCAA.

Materials and Methods

Cell Culture: Non-malignant, transformed human mesothelial cells, Met-5A (ATCC, Manassas, VA) were grown in RPMI medium (CellGro Mediatec, Manassas, VA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and antibiotics. Cells were maintained at 37° C and 5% CO_2.

Human serum samples: Serum samples were collected by the Center for Asbestos Related Disease (CARD) in Libby, Montana in accordance with Idaho State University IRB project approval #3292MOD and were stored at -80°C until needed. Samples previously identified as MCAA positive (+ive) or negative (-ive) were pooled (Marchand et al., 2012) and small aliquots stored at 20° C. Sera cleared of IgG antibodies were used as negative controls. Sera were cleared using Protein A Agarose Beads (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. IgG removal was confirmed by running sera on a

12% Bis–Tris gel (Novex, Life Technologies, Carlsbad, CA) and staining with GelCode Blue Stain Reagent (Thermo Scientific) and checking for lack of bands corresponding to the molecular weight of IgG heavy and light chains (50 and 25 kDa).

Biotin Labeling and Affinity chromatography: Cells were grown in T-75 flasks until ~85% confluency was reached. The cells were rinsed with 2 washes of ice cold PBS for no more than 5 seconds each wash. The cells were then covered with Pierce sulfo-biotin labeling solution (Pierce Cell Surface Protein Isolation Kit, Life Technologies, Grand Island, NY) and agitated for 30 minutes at 4° C. The reaction was stopped by adding 500 uL of quenching solution to each flask. The cells were then scraped into suspension and centrifuged at 500Xg for 3 minutes. The flasks were rinsed with 10 mL 25 mM Tris Buffered Saline (TBS) and the cells removed were added to the total cell pellet.

The cells were lysed for 30 minutes at 4° C using lysate buffer treated with protease inhibitor cocktail (Pierce) and sonicated 5 times for one second each at 40 kHz (Branson Ultrasonic, Danbury CT). The lysate was placed in an ice bath for 30 minutes and was sonicated every 5 minutes for 1 second to improve solubility. The lysate was then clarified by centrifugation at 10,000Xg for 2 minutes. The supernatant was transferred to a fresh tube.

The lysate was then added to a freshly washed neutravidin agarose affinity column. This was incubated at 25° C under constant rotation for 60 minutes. The column was washed using the kit's wash buffer treated with a protease inhibitor cocktail (Pierce). The column was then washed three times by adding protease

inhibitor treated wash buffer and centrifuging for 2 minutes at 1,000 g. The protein was eluted from the column by reducing the disulfide bond in the biotin tag using 50 mM DTT at 25° C for 60 minutes. Protease inhibitors were then added to the eluate. The lysate was kept at 4° C until it was added to the size exclusion column.

Immunoprecipitation: The IgG from MCCA positive serum was bound to protein G beads as per manufacture's instructions. The beads were then washed 3 times with PBS. The beads where then incubated overnight under constant rotation with 400 uL of protein lysate obtained using the biotinylation kit. The protein was eluted from the beads by boiling them in SDS loading buffer for 5 minutes. The proteins were separated on a Bis/Tris gel following a standard SDS PAGE protocol. All bands were cut out from the gel and prepared for Ms/Ms analysis.

Antigen specific clearing of MCAA+ serum: A commercially isolated form of a candidate antigen was biotinylated in 360 uL of 5mM SS biotin in PBS at 4° C for 2 hours under constant rotation. 50 uL of quenching solution was added to the reaction. The solution was cleaned of labeling reagent using a Pierce 10 kDa concentration unit spun at 14,000 rcf and resuspended in 360 uL of 25 mM Tris buffered saline. The solution was then added to 250 uL of neutra avidin bead suspension (Pierce Cell Surface Protein Isolation Kit, Life Technologies, Grand Island, NY) and kept under constant rotation for 1 hour at 25° C. The column was spun at 1,000 rcf for 2 minutes to remove excess solution. The column was washed two times with 500 uL of 25 mM tris buffered saline and spun out. Finally the column was capped, and loaded with 250 uL of 25 mM tris buffered

saline and 105 uL of MCAA+ serum. This was kept under constant rotation at 4° C for 12 hours. The serum was collected from the column by uncapping the bottom and spinning at 1,000 rcf for 2 minutes. The serum was concentrated and washed two times with 500 uL PBS by spinning in a 10kDa concentrator. After the final wash the serum was returned to original concentration by adding enough PBS to bring the volume to 105 uL.

Dot Blot: 10 uL of solution containing surface proteins was dotted on a PVDF membrane wetted in methanol and allowed to dry. The membrane was then rewetted in methanol and blocked for 1 hour in 5% non-fat dry milk in PBS buffer. The membrane was then incubated overnight in 3% BSA/PBS containing MCAA positive serum at 1/1000 dilution. The membrane was then washed for four minutes with 0.1% Tween. This step was repeated two more times. The membrane was then blocked again for 1 hour in 5% Milk buffer. The membrane was then incubated for one hour in 3% BSA/PBS containing a goat antihuman IgG. The membrane was again washed three times with 0.1% Tween. The membrane was then developed in Pierce 1 step TMB solution (Life Technologies). The development was stopped by placing the membrane in PBS. The membrane was then covered with a paper towel and allowed to dry. The membrane was imaged using a Versa Doc (BioRad, Hercules, CA) imaging system. A control for the presence of protein was performed by staining with Ponceau S. for 5 minutes and then destaining with water until protein is spot is visible.

Size Exclusion Chromatography: Surface protein lysate/suspension (1 mL) was loaded into a U.N.I.C.O.R.N. linked to a GE 16/60 Sephacryl S-100 HR size exclusion column equilibrated in PBS. PBS was pumped through the column at a rate of 0.5 mL/min. 1.5 mL fractions were collected in autoclaved test tubes and transferred to a 1.5 mL centrifuge tube on ice within 15 minutes of eluting from the column. The total number of fractions collected was 143.

BCA analysis of Fractions: Protein concentration was determined using a Pierce BCA kit. The screening of fractions 1 through 70 was optimized to detect between 5 ug-250 ug by placing it at 60° C for 30 minutes. The rest of the samples were processed at 37° C.

Protein Lysate ELISA: 200 ng of protein from each sample, as determined by BCA assay (Pierce), were placed in 100 uL of carbonate coating buffer in wells of a polystyrene high affinity 96 well plate and left overnight at 4° C. The buffer was washed off using 200 uL of PBS per well. This was repeated three times. The plates were then blocked in 200 uL of 5% non-fat dry milk in PBS for 1 hour at 25°C. The blocker was then aspirated off twice and the plates were incubated with 100 uL buffer containing MCAA positive serum diluted at a ratio of 1/1000 in 3% BSA overnight at 4° C. The next day, the plates were washed three times with PBS, waiting minutes between washes. The plates were then incubated with Goat antihuman IgG HRP for 1 hour at 37° C. The plates were washed a second time using the same steps as previously described. The plates were then developed using 100 uL of Pierce one-step TMB ELISA developer. Development was stopped by adding 50 uL of 1 M HCl. Plates were analyzed at 450 nm on a

microtiter plate reader (BioTek Instruments, Winooski, VT). Non-specific secondary antibody binding was corrected for on a plate-to-plate basis by subtracting the mean optical density (OD) for the secondary antibody-only control wells from the mean OD of each sample.

SDS-PAGE: 20-30 ug of protein were loaded into a NuPage 4-12% Bis/Tris gel. Gels were run at a constant voltage of 185 V for 60 minutes. The gel was then removed from its casing and stained using either a BioRad Zinc Stain kit or Coomassie Blue stain.

In solution tryptic digestion: 5 uL of 1 M ammonium bicarbonate (pH 8.0) was added to every 45 uL of protein suspension. 5.5 uL of 10 mM DTT was added to every 50 uL of this solution and the reaction was placed in a heat block set at 50° C for 15 minutes. The reaction was cooled to room temperature. 13.9 uL of iodoacetamide was added to every 55.5 uL of reaction solution and the solution was allowed to react at room temperature in the dark for 20 minutes. Proteomics grade Trypsin (Sigma Aldrich) was added at a 1:100 dilution. The samples were incubated at 37° for 16 to 20 hours. Acetic acid was added to acidify and stop the trypsin digestion.

Desalting and Shipping: Samples were desalted using a C18 ziptip (Millipore). The procedure was done according to the manufacturer's instructions. The eluted sample was then placed in a SpeedVac and evaporated to dryness. Samples were then shipped to the Mass Spectrometry CORE facility at the University of Idaho, Moscow ID.

Detection of Serum Antibodies to plasminogen: Purified human plasminogen (R&D Systems) was coated to high binding ELISA plates in carbonate coating buffer (pH 8) overnight. After blocking with 5% non-fat dry milk for 2 hr, serum samples (diluted 1:100 in 3% BSA in PBS) from LA-exposed MCAA positive and negative serum samples were added to wells in duplicate, as were serum samples from healthy controls (PrecisionMed, Solana Beach, CA) that had been shown to be MCAA negative by cell based ELISA as previously described [7]. After 2 hr at room temperature, the wells were washed in PBS-Tween and then stained with anti-human IgG HRP antibodies for 1 hr, followed by development using TMB substrate. The optical density of the wells was analyzed at 450 nm using the BioTek plate reader. The data are reported as the number of standard deviations above the mean of the absorbance values for wells with known negative controls.

Bioinformatic searches: The STRING 10 protein interaction network was selected to analyze potential connections to ECM deposition or degradation because it provides weighted connections within the generated networks [13]. Searches were performed by using the specific names of candidate genes, eg., COL1A1, and the key words 'collagenase,' 'collagen'. All return hits were included in the network. The network was then reduced to only those proteins that had a connection to one of the protein candidates and COL1A1.

Cell Based ELISA for detecting Collagen: Human Met5a cells were plated into a 96 well plate at 70,000 cells per well. The plates were incubated at 37°C for 3 hours. The cells were then treated according to their treatment group designation with

an n=6. The cells were then incubated for 82 hours at 37° and then rinsed once with PBS. All blocking and incubation steps were performed under agitation at room temperature for 1 hour with washing steps occurring immediately following incubation steps. The wells were then blocked with 200 uL 5% non-fat dry milk in PBS. The wells were incubated with 2 ug of mouse IgG targeted to collagen type 1 (Abcam, Cambridge, MA) in 100 uL of 3% BSA. The plates were then washed 3X with 200 uL of 0.05% Tween in PBS for 4 minutes each wash. The plates were then blocked again with 5% Milk in PBS. The plates were then incubated with goat anti-mouse IgG conjugated to HRP (Life Technologies). The plates were then washed 3X with 200 uL of 0.05% Tween in PBS. The plates were then developed with 100 uL of Pierce One Step TMB ELISA reagent. The reactions were stopped with 50 uL of 1M HCL. The plates were then read for absorbance at 450 nm on a plate reader (BioTek Instruments, Winooski, VT).

Cell Based ELISA for detecting MCAA binding: A cell based ELISA was used a previously described[8]. In brief, Met5a cells were seeded at 100,000 cells per well in a 96 well plate. The cells were allowed to adhere overnight at 37° C and then fixed in 1% Para formaldehyde. The plates were blocked for 1 hour at room temperature in 5% nonfat Milk in PBS. The cells were then exposed to either serum that had been identified as MCAA+ or MCAA+ serum that had been cleared of selected components. The serum had been diluted in 3% BSA in PBS at a 1/1000 ratio. The cells were incubated at room temperature for 1 hour and then washed for 4 minutes in 0.05% PBS Tween. This was repeated three times. The plate was then blocked a second time in 5% nonfat Milk in PBS for 1 hour.

The wells were then incubated with 100 uL of 3% BSA PBS containing a goat antihuman IgG secondary that had been conjugate to an HRP molecule, diluted at 1/1000. The plate was again incubated under agitation for 1 hour at room temperature. The plate was then washed for four minutes in 0.05% PBS Tween, repeated three times. The plates were then developed with 100 uL of Pierce One Step TMB ELISA reagent. The reactions were stopped with 50 uL of 1M HCL. The plates were then read for absorbance at 450 nm on a plate reader (BioTek Instruments, Winooski, VT).

Statistical Analysis: One and Two way ANOVAs were run using StatPlus (StatPlus Software, Walnut CA). Students t-tests between two populations were run using Excel. Statistical significance was defined as P values <0.05. Data are graphed with error bars indicating SEM.

Results

It was seen that $4x10^7$ cells yielded ~3.5-4 mg of total protein for every two biotinylation procedures. To test for the presence of epitopes bound by MCAA, we made comparisons between western blot and dot blot procedures. The results revealed marks only on the dot blot, showing this is where binding by MCAA had occurred (Figure 2.1). This indicated that the linearization of the proteins in preparation for SDS PAGE was destroying the epitope. This provided the rationale for using native techniques in the remainder of our attempts.

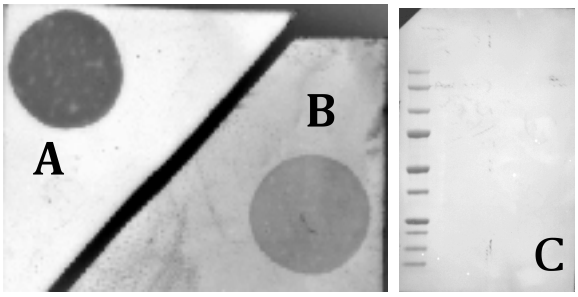

Figure 2.1. MCAA binding of Dot blot compared to MCAA Binding of Western Blot: 10 uL of isolated surface protein lysate was blotted onto each piece of a PVDF membrane. A) One membrane was stained with Ponceau S. stain B) The second piece was imaged using the dot blot procedure as described in the methods. C) The protein lysate was run out in a gel via SDS PAGE and transferred to a PVDF membrane. The membrane was treated as described in the staining of the dot blot procedure.

The first technique we attempted to isolate MCAA was immunoprecipitation of cell lysates following extraction of biotinylated surface proteins using MCAA positive serum to capture the targets, followed by MS/MS analysis. The initial results of MASCOT search showed high concentrations of cytoskeletal keratin 9 and 10 in a majority of the samples (data not shown). This implicated that contamination by intracellular proteins had occurred during preparation and that the signal to noise ratio was too low to effectively identify any potential integral membrane receptor targets. Based on this data, it was determined that size exclusion chromatography had the potential to yield more effective results.

Of the protein that was added to the column, ~18.3% came off between fractions 17 and 70 (Figure 2.2). The next ~79.1% came off between fractions 71 and 99. The remaining ~2.6% came off between samples 100 and 143. The majority of the protein was eluted from the column prior to fraction 94. The greatest amount of isolated protein was eluted in fraction 81. The results of the MCAA binding assay, shown in Figure 2.3, show that binding was statistically

highest in samples 27 and 42 (Figure 2.3). When the data from these two figures are considered together, it indicates the protein targets of MCAA are low abundance proteins as binding was highest in dilute samples were protein complexity was likely to be lower (Figures 2.2 and 2.3).

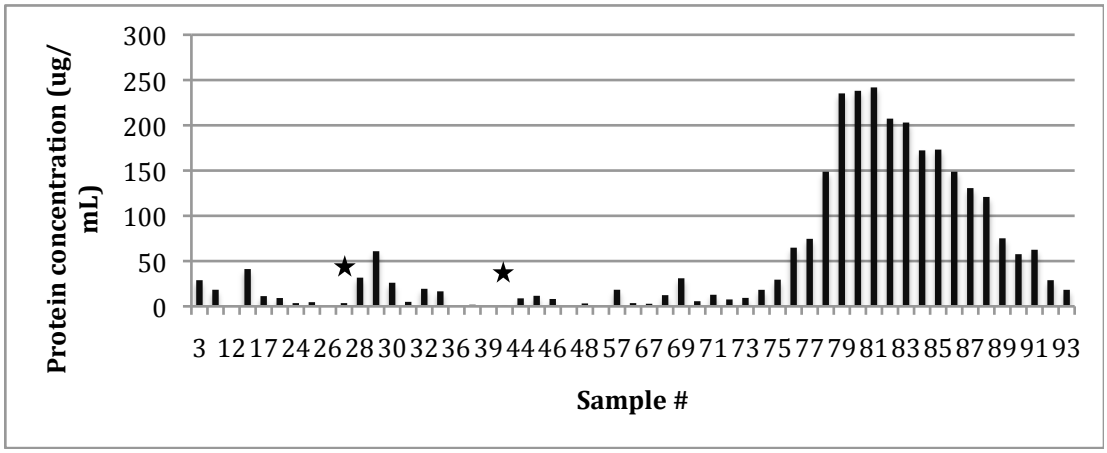

Figure 2.2 BCA assay of fractions collected from the size exclusion column: Protein concentration was obtained using a Pierce BCA assay kit. A calibration curve was created using BSA dilutions (r^2 of >0.96), concentrations were determined by back calculating against the reference curve. Samples that possessed high binding(indicated by ★) possessed relatively low protein concentration.

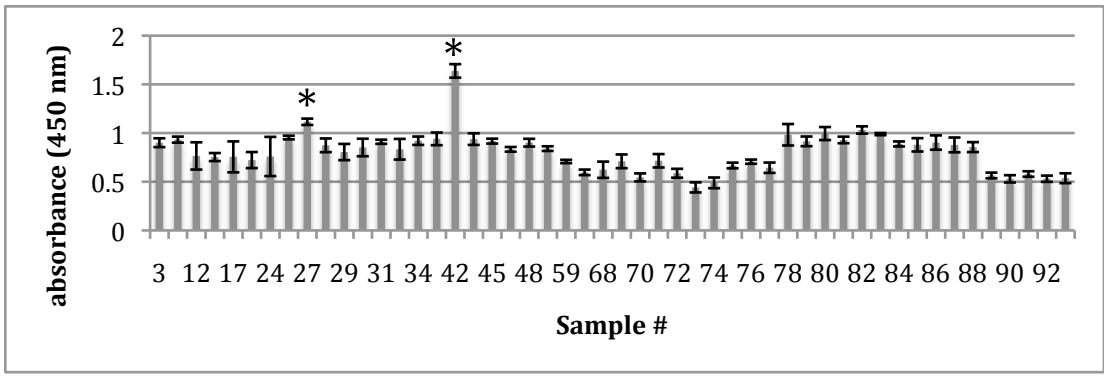

Figure 2.3 Protein lysate ELISA: The fractions with sufficient protein were then analyzed using a protein lysate ELISA protocol. Samples were prepared in triplicate and analyzed by absorbance on a plate reader set to 450 nm. Standard error of the mean was calculated; and samples were considered of interest if they had an absorbance higher than 1 *=$p<0.01$ by Students t-test.

The MS/MS spectra obtained from in solution digestion of fractions 27 and 42 was processed using the Protein Lynx Gobal Server V2.3 (Waters Corp). The peak list files were analyzed via the MASCOT public database. In Sample 27 plasminogen received a score of 30, the highest score received in that search, indicating homology (Table 2.1). It was also found in sample 42 with a score of 34. Fraction 30 was also analyzed to provide information about the proteins contained in low binding samples. None of the key identified proteins in the high binding samples were found (data not shown).

Table 2.1. Proteins isolated via Size exclusion chromatography

Fraction #	Identified Protein	NCBI accession no.	Expected/ Calculated mass (Da)	% sequence coverage	Mascot Score	Peptide Sequence
27	plasminogen	AAH60513	1045.5342/ 1045.5291	1%	30	K.LSSPADITDK.V
42	plasminogen	AAH60513	1045.5434/ 1045.5291	1%	34	K.LSSPADITDK.V

Samples were examined on a Water's ESI-QUAD-TOF Mass spectrometers. Peaks lists were analyzed using MS/MS ion search. The Mascot score is a probability based MOWSE score. The ion score is -10Xlog(P) where P can be understood as the probability that an observed match is a random event. Scores >22 for sample 27 and >38 for sample 42 indicate homology (p<.05). Protein scores are inferred from ion scores as a basis for ranking hits.[15]

The fact that a plasminogen homolog was identified in both samples that contained high binding from MCAA and not in the fraction with lower binding motivated us to test the binding of MCAA to plasminogen. The data from experiments testing the binding of MCAA to plasminogen implicate some striking conclusions. The mean absorbance of samples bound with antibodies from serum containing MCAA was 3 times higher ($p < 0.01$) than the absorbance of MCAA negative samples and normal human serum (Figure 2.4A). In addition, 88% of the MCAA-positive samples showed binding to plasminogen, suggesting

that anti-plasminogen antibodies are present in most MCAA-positive patients exposed to LA. This percentage was significantly higher than that seen in serum from MCAA-negative patients or normal human serum (Figure 2.4B).

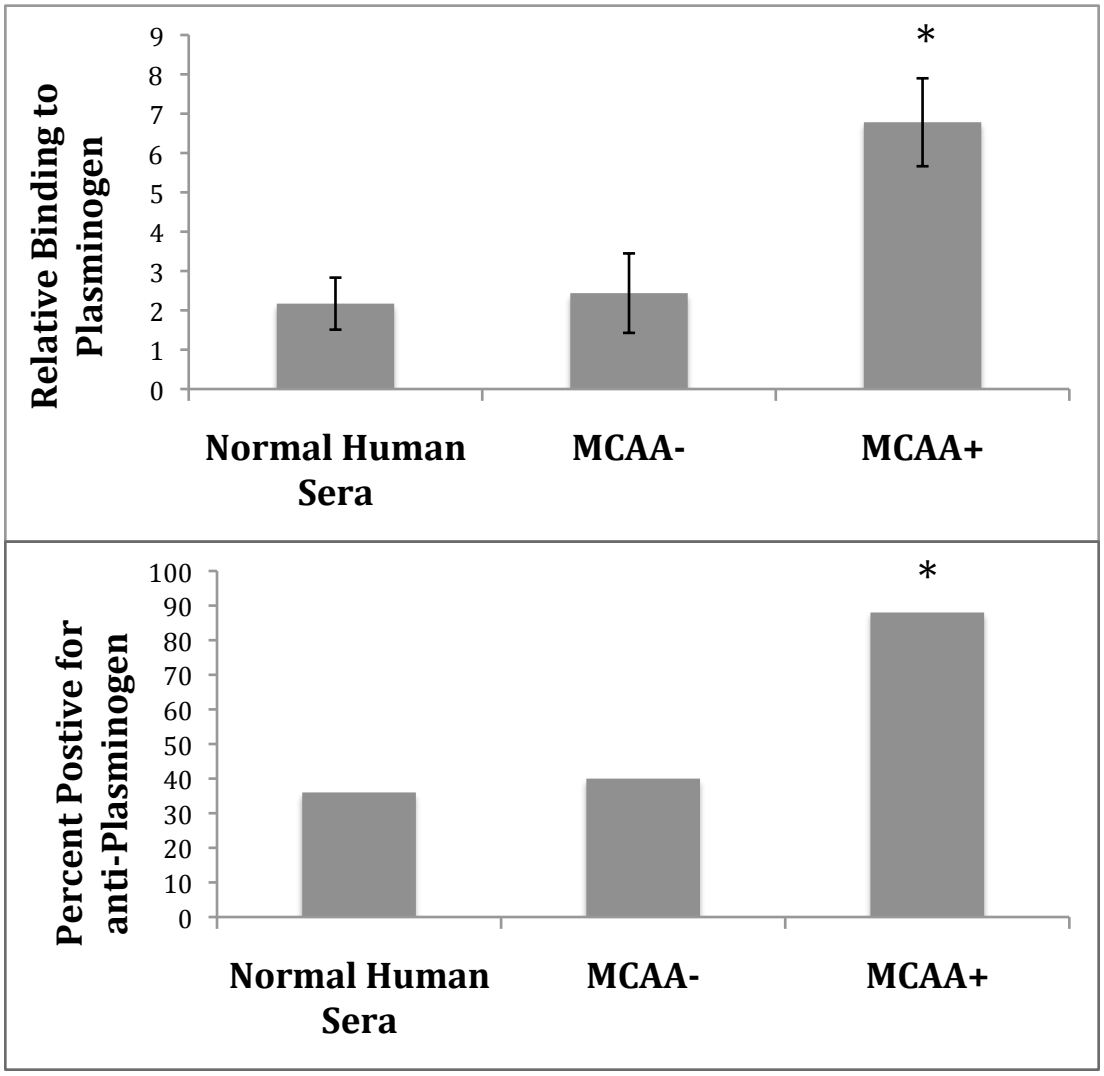

Figure 2.4. Binding of Plasminogen by MCAA via ELISA: Plasminogen was bound in a 96 well plate and various treatment groups were treated as described in the ELISA protocol. Normal Human Serum was obtained from Precision Med(Solana Beach, CA). The other two groups were treated with serum taken from the population of Libby Montana. The plate was read at 450 nm on a BioTek microtiter plate reader. Top: Data indicate the average number of standard deviations above the mean absorbance for known negative control sera. N=11-20 in each subset, * = $p < 0.01$ by one-way ANOVA and by two-tailed t-tests comparing to Normal Human Sera. Bottom: The percent positive for anti-plasminogen among the subsets of serum samples. N = 11-20 in each subset, * = $p < 0.01$ by Fisher's Exact Test.

With the evidence produced implicating the involvement of the plasminogen/plasmin system, the data obtained from analyzing samples purified via immunoprecipitation was reexamined for surface bound proteins. Of the 21 bands analyzed; bands 2, 4, and 1a held fragments of interest as determined by MASCOT. These three all implicated the presence of proteins that were involved in the regulation of ECM. Of these, two were surface proteins (cytoskeletal keratin 18, annexin A5) (Table 2.2).

Table 2.2 proteins isolated via immunoprecipitation

Identified Protein	NCBI accession ID	Expected/ Calculated mass (Da)	% sequence coverage	Mascot Score	Fragment Sequence
Annexin A5	ANXA5_HUMAN	1702.8818/ 1703.8941	19%	282	K.GLGTDEESILTLLTSR.S
Keratin, type I cytoskeletal 18	K1C18_HUMAN	806.3786/ 806.3923	3%	70	R.LAADDFR.V

Samples were examined on a Water's ESI-QUAD-TOF Mass spectrometers. Peaks lists were analyzed using MS/MS ion search. The Mascot score is a probability based MOWSE score. The ion score is -10Xlog(P) where P can be understood as the probability that an observed match is a random event. Scores >46 indicate identity (p<.05) Scores >32 indicate homology. Protein scores are inferred from ion scores as a basis for ranking hits.[14]

The searches performed using STRING 10 showed evidence that plasminogen interacts with at least 9 proteins that also interact with COL1A1 in both *homo sapiens* and *mus musculus*[15] (Figure 2.5). Of these, the three with high confidence functional links to both plasminogen and Collagen were MMP-9, MMP-2, and ITGA2 (Tables 2.3 and 2.4). It was found that cytoskeletal keratin 18 had a medium confidence interaction with plasminogen. The most direct path with the highest score was between plasminogen and MMP9 with a score of 0.998. MMP9 connected to COL1A1 with a score of 0.811. Since MMP9 is a collagenase that is activated by plasminogen, this could serve as a possible mechanism for the collagen deposition observed in previous studies [8].

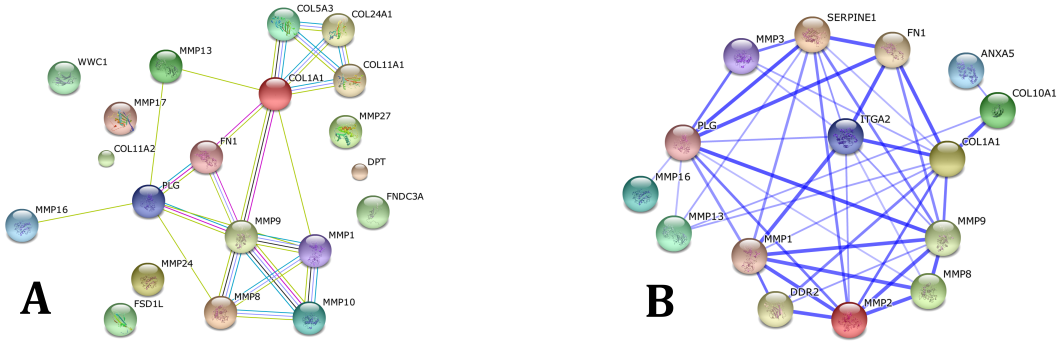

Figure 2.5 STRING network: A brief search was performed using the STRING database to test the validity of the tandem mass spectrometry data. (A) Network view based on types of published data available. (B) Network vie w based on confidence of the data in the current literature[13].

Table 2.3 Summary of STRING network

Protein Symbol	**Function of interest**	Interaction with PLG score (HUM)	Interaction with Col1a1 score (HUM)	Interaction with PLG (MUS)	Interaction with Col1a1 score (MUS)
MMP9	Collagenase: breaks down Collagen type I, II, and III	0.998	0.811	0.923	0.638
MMP2	Collagenase	0.801	0.857	0.525	0.648
ITGA2	Integrin: interacts with collagen and up-regulates genes that lead to modification of the ECM	0.586	0.985	0.629	0.481
MMP3	Collagenase	0.848	0.517	0.622	0
MMP1	Collagenase	0.632	0.549	0.622	0
MMP13	Collagenase	0.414	0.546	0	0.829
KRT18	Cytoskeletal keratin 18, surface receptor, implicated with moving mutated cystic fibrosis transmembrane receptor to the cell surface	0.64	0	0.254	0
DDR2	Receptor that interacts with collagen, upregulates expressions of MMP9	0	0.746	0	0
Annexin A5	Shields lipids that interact with coagulation processes.	0	0	0	0

This table summarizes the strength of evidence for interactions between key proteins and plasminogen (PLG) and type 1 collagen (Col1a1) in both human (HUM) and murine databases. The interaction scores were determined by an algorithm that utilizes evidence based on genomic location, gene fusions, concurrence across genomes, co-Expression, experimental/biochemical data, association in curated databases, and co-mentions in Pub-Med abstracts to calculate the final score.[13]

Table 2.4 Interactions of DDR2 and ITGA2

	Interaction score with DDR2 (HUM)
COL1A1	0.746
MMP9	0.574
MMP1	0.886
MMP2	0.993
	Interaction score with ITGA2 (HUM)
COL1A1	0.985
MMP9	0.581
MMP1	0.904
MMP2	0.56
MMP8	0.4

This table summarizes the strength of evidence for interactions between the collagen receptors DDR2 and ITGA2 and key genes within the network. The interaction scores were determined by an algorithm that utilizes evidence based on genomic location, gene fusions, concurrence across genomes, co-expression, experimental/biochemical data, association in curated databases, and co-mentions in Pub-Med abstracts to calculate the final score[13].

To determine whether PLG targeting MCAA had an activating or inhibiting effect on PLG, we compared the collagen deposition induced by a commercial antiplasminogen antibody (R&D Systems) that inhibited PLG activity to the deposition induced by exposure to MCAA. We found that both treatments increased collagen deposition higher than that of cells treated with cleared serum. (Figure 2.6) Statistical significance was determined via t-tests with p values of 3.4E-4 and 1.1E-5 respectively.

To discover whether or not other proteins were being targeted by MCAA, we compared binding of un-manipulated MCAA+ serum to Met5a cells with binding observed by serum that had been cleared of PLG targeting antibodies and serum that had been cleared of all IgG. The results of this cell based ELISA showed that the binding of serum cleared of PLG targeting antibodies was statistically lower than whole MCAA+ serum (p=3.6E-7) and statistically higher than serum cleared of IgG (p=4.1E-8). This indicated that other subsets of MCAA existed which target different proteins on the Met5a cell.

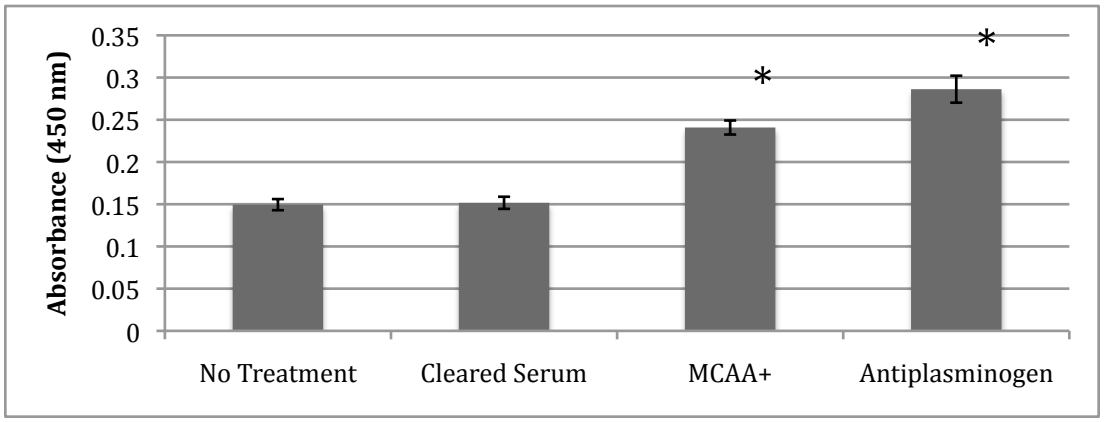

Figure 2.6 ELISA demonstrating the role of plasminogen in collagen deposition: The collagen deposition induced by MCAA+ serum was compared to the collagen deposition induced by a commercial antihuman plasminogen antibody. Error bars indicate Standard error of the mean. A one way ANOVA indicated that differences were statistically significant.

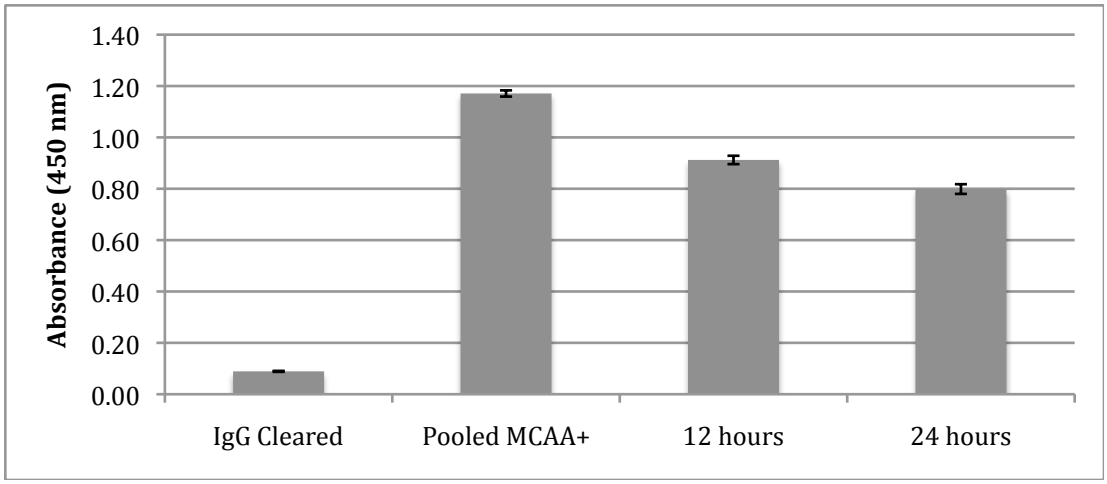

Figure 2.7 Binding of MCAA+ cleared of plasminogen targeting antibodies: Serum that had been cleared of antibodies that targeted human plasminogen (PLG) were tested for MCAA+ binding to surface proteins on mesothelial cells. The binding of two samples that had been cleared for different times (12 hours and 24 hours) were compared to binding of serum cleared of all IgG and un-manipulated MCAA+ serum. The binding of PLG cleared serum was higher than IgG cleared, but less than that of MCAA+. The Difference between groups was confirmed via t-test with a p-values <0.05 for IgG vs. 12 hours, Pooled MCAA vs. 12 hours, and 12 hours vs. 24 hours.

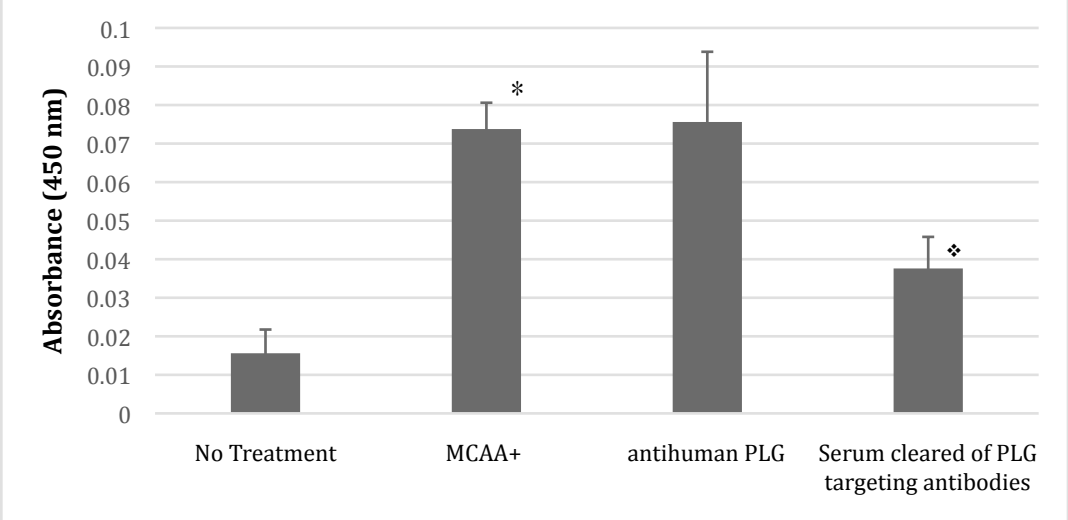

Figure 2.8 Collagen Deposition in serum cleared of PLG targeting MCAA: To see how much of the collagen deposition induced by MCAA could be attributed to the presence of the PLG targeting MCAA subset, the collagen deposition induced by MCAA+ serum was compared to that induced by MCAA+ serum that had been cleared of PLG targeting antibodies. The observed difference between MCCA+ and No treatment was statistically significant when analyzed via a t-test *=p<0.05. The collagen deposition of Serum cleared of PLG targeting antibodies was also deemed to be significant via a t-test ❖=p<0.05

To approximate how much of the induced collagen deposition could be accounted for by the PLG targeting MCAA, we compared the collagen deposition induced by whole MCAA+ serum to that induced by serum that had been cleared

only of PLG targeting MCAA antibodies (Figure 2.8). We found that the collagen was reduced and that the results were statistically significant (p=0.001).

Discussion

Exposure to LA asbestos drives production of various autoantibodies as well as a progressive form of pleural fibrosis termed LPT. The potential for autoantibodies to exacerbate fibrotic disorders has been suggested by others [11,12,16]. In those cases, primarily in systemic sclerosis, the fibrosis occurred in the lung and the mechanism was hypothesized to be binding of anti-fibroblast antibodies (AFA) to surface receptors such as Platelet-Derived Growth Factor Receptor (PDGF-R) that play a role in collagen deposition. However, for pleural fibrosis, the primary disease outcome from LA exposure, it seemed more likely that the autoantibodies would target mesothelial cells that line the pleural cavity. In support of this hypothesis in the case of the Libby LPT, we have previously demonstrated the presence of autoantibodies to mesothelial cells (MCAA) in LA-exposed subjects, as well as an association between the presence of MCAA and pleural fibrosis[7]. However, the mechanism whereby these autoantibodies would induce or exacerbate pleural fibrosis is unknown.

In order to begin to understand that mechanism, this study was designed to use proteomics approaches to identify potential cell surface targets for the MCAA. Surface proteins were collected by biotinylating the extracellular domains on whole cells, and then extracting from the whole cell lysates via an avidin affinity column. The resulting proteins were separated into size fractions,

which were probed for binding using an ELISA format. High binding fractions were then analyzed by MS/MS.

Two significant challenges exist when identifying the protein target of an antibody when they are located in the plasma membrane of a cell. First, the surface of a cell has a massive variety of proteins filling a variety of roles; far too many to test each protein individually[17]. Thankfully, in the last three decades techniques such as mass spectrometry have become sophisticated enough that they may be applied to problems involving proteomics[18,19].

The second challenge encountered in identifying a protein target is to obtain a sample that is pure enough. Surface receptors generally have three specific domains: extracellular, hydrophobic, and cytosolic. This amphipathic aspect makes it difficult to keep them in solution or to even liberate proteins from the plasma membrane in the first place[20]. Often, classic techniques would achieve the goal of keeping plasma membrane proteins in solution, but the samples were filled with too many contaminants from intracellular membranes to obtain clear data. In recent years, techniques such as biotinylation have allowed researchers to label surface proteins and purify them using affinity chromatography, removing the majority of contaminating proteins[18].

Fractions 27 and 42 from the size exclusion separation were selected for MS/MS analysis because they displayed the highest statistically significant absorbance when probed for MCAA binding. This indicated that an epitope for MCAA made up a larger percentage of the proteins in these fractions. Therefore,

the MS/MS profiles of the proteins in these samples would provide higher quality data for protein inference.

The top 50 hits of each MASCOT analysis were examined for surface proteins. Of these, plasminogen was one of two proteins that appeared in both samples and had the highest MASCOT score in both reports with MASCOT scores of 30 and 34. A review of the literature revealed a precedent with regards to the role of plasminogen in fibrosis. Activation of the plasminogen/plasmin system has been shown to slow progression of fibrosis in experimentally induced asbestos disease for murine models [21,22]. Deficiencies in plasmin or plasminogen activator have led to increased fibrosis[23]. Therefore, autoantibody binding that inhibited this system would be expected to increase fibrotic collagen deposition.

The next logical step was to test for binding of the MCAA to plasminogen to demonstrate the presence of anti-plasminogen autoantibodies. The mean absorbance from the binding ELISA using commercial purified plasminogen was 3 times that of the control groups as seen in Figure 2.5. This shows that plasminogen is the most likely candidate for our target protein isolated from the size exclusion method.

The STRING 10 protein interaction network was selected to analyze potential connections to ECM deposition or degradation because it provides weighted connections within the generated networks [13]. When connections between plasminogen/plasmin system and collagen type 1 were searched for using the STRING database, multiple two-node pathways were found between

plasminogen and Collagen type 1 in both *homo sapien* and *mus musculus* [15]. Table 2.3 outlines the details of our findings. The most noteworthy connection was between plasminogen, MMP9, and Collagen Type 1. Since MMP9 is a collagenase that is activated by plasminogen, this could provide a possible mechanism for the collagen deposition observed in previous studies [8].

It is interesting to note that two tyrosine receptor kinases (TRK), which contribute to governing ECM composition, interact with collagen. Discoidin domain containing receptor 2 (DDR2) and ITGA2 both assist in collagen regulation and are implicated in the upregulation of matrix metalloproteases [15]. It is possible that an imbalance in the degradation of collagen would result in an upregulation of these pathways to increase production of MMPs in their inactive form. While there is no evidence yet supporting a role in pleural fibrosis, involvement in lung fibrosis has been demonstrated and a loss of DDR2 results in promotion of hepatic fibrosis. [24,25].

We also felt that it was important to answer three other questions regarding the involvement of PLG in the excess collagen deposition observed: We wanted to know if MCAA was activating or deactivating PLG; we wanted to know if PLG was the only protein being targeted by MCAA; and we wanted to know approximately how much of the excess collagen deposition could be attributed to the binding of PLG by MCAA.

To investigate if MCAA binding was activating or deactivating plasminogen, we exposed Met5a cells to a commercial antihuman plasminogen

antibody that inhibits plasminogen activity. The results (Figure 2.6) show that collagen Type 1 deposition was upregulated from that of cleared serum ($p=1.17E-5$); suggesting that the subset of MCAA which target plasminogen inhibits its activity.

To test if PLG was the only protein on Met5a cells targeted by MCAA, we performed a cell based ELISA which compared the binding of whole MCAA+ serum to that of serum that had been cleared of PLG targeting antibodies. The results showed that the binding of serum cleared of PLG targeting antibodies was less than that of whole serum ($p=0.001$) but greater than binding of serum cleared of all IgG ($p=4.1E-8$). This indicates that there are other proteins being targeted by other subsets of MCAA.

One limitation of this study occurred because of a limited number of MS/MS samples. Due to time constraints we only sent samples that had a reasonable expectation of holding a protein target that had been bound by MCAA. Because of this, we never validated the efficiency of our recovery of plasma membrane protein. Though it was evident from the samples we did send that some plasma membrane proteins had been extracted, we did not obtain a total yield. As a consequence, some potential targets may not have been liberated from the plasma membrane and would not have been detected via the binding of MCCA. The data from Figure 2.6 clearly shows that MCAA has subsets of antibodies that target proteins that are not plasminogen. Further studies are warranted to identify other potential targets.

Our study has demonstrated that it is reasonable to assume that multiple targets could be bound by MCCA. cytokeratin 18, a surface protein that was identified in the results, interacts with components of the plasminogen system. It has been linked to pulmonary fibrosis and cystic fibrosis [26,27]. Binding by MCAA on all of these targets could lead to alterations in multiple regulatory mechanisms which could result in either opposing or synergistic effects with regards to the accumulation of collagen in the ECM.

The second protein identified, annexin A5, also identified as a potential MCAA target, plays a role in shielding key surface lipids from participating in the process of coagulation[28–30], and exacerbates silica-induced pulmonary fibrosis through an unknown mechanism[31]. Interestingly, annexin A5 binds avidly to phosphatidylserine, a plasminogen receptor [32]. Anti-annexin A5 antibodies might therefore play a role in altering the surface binding of plasminogen. Surface bound plasminogen is much more active than free plasminogen [33], so an antibody that prevented surface binding may also increase fibrosis.

Our final experiment was designed to test the most intriguing finding from our MS/MS procedure. We wanted to see whether or not any of the collagen deposition induced by MCAA+ serum could be attributed to the subset of PLG targeting antibodies. To do this, we compared the collagen deposition induced by whole MCAA+ serum to that of serum that had been cleared of PLG targeting antibodies. Our results showed that the collagen deposition was significantly reduced ($p=0.001$). This suggests that PLG is a target of MCAA and that this interaction has the ability to contribute to increased collagen deposition

in Met5a cells as observed by Serve [8]. This mechanism could contribute to the accumulation of scar tissue near epithelial cells as it is seen in LPT.

Future work

The first step to further this work could be to confirm the role of plasminogen in the excess collagen deposition as it has been observed in a murine model. Since maintenance of the ECM involves many regulatory systems, it is important to confirm that the manipulation of one system is enough to lead to the observed symptoms. This would be critical in the development of therapeutic strategies based off a true understanding of the pathogenesis of LPT.

Conclusion

We were able to show that a subset of MCAA preferentially bind plasminogen**.** We have also shown that it is likely that MCAA binding to plasminogen contributes to a sizable portion of the excess collagen deposition seen in Met5a cells exposed to asbestos-induced MCAA. We also showed that the collagen deposition induced by MCAA+ is similar in nature to the collagen deposition induced by an antihuman plasminogen antibody. This suggests that the MCAA which target plasminogen inhibit its activity. These results provide strong evidence for a developing a mechanism of pathogenesis by MCAA. It also provides a good foundation for identifying possible therapeutic targets for those suffering from LPT.

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Chapter 3

Pleural Collagen Deposition Following Libby Amphibole Exposure Involves Autoantibody-Induced Protein Tyrosine Phosphorylation

Pleural Collagen Deposition Following Libby Amphibole Exposure Involves Autoantibody-Induced Protein Tyrosine Phosphorylation

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Abstract

Asbestos induced Lamellar Pleural Thickening (LPT) is an emerging disease with the potential to affect thousands of lives through environmental exposures to amphibole asbestos. Unlike typical pleural plaques, LPT is progressive and results in pulmonary function decline and death. Many details of the mechanism of pathology remain unknown, but a key factor in the collagen production may be the presence of mesothelial cell autoantibodies (MCAA) that have been generated in response to asbestos exposure. Excessive collagen deposition into the extracellular matrix (ECM), a key event in the development of fibrosis, has been observed in cell cultures exposed to these antibodies, along with activation of matrix metalloproteinases (MMP).

The focus of this study was to identify the cellular signaling pathways affected by these antibodies, with the long-term goal of identifying therapeutic targets for intervention. Using flow cytometry, and cell-based ELISA, we have demonstrated that increased tyrosine phosphorylation occurs in mesothelial cells (Met5a) exposed to amphibole asbestos-induced MCAA. This is consistent with other studies demonstrating a role for tyrosine phosphorylation in fibrotic pathways and MMP activation. Serine and threonine phosphorylation were also examined, but statistically significant changes were not observed. Inhibition of tyrosine phosphorylation with Genistein inhibited collagen formation by mesothelial cells treated with MCAA. These data will influence future efforts in characterizing the signaling pathways and identifying the target receptor for MCAA.

Introduction

Lamellar Pleural Thickening (LPT) as it is seen in the town of Libby, MT has been defined by the presence of a diffuse thickening of the pleural lining surrounding the lungs. This has resulted in many of the residents of Libby suffering from a severe reduction in pulmonary function[1,2]. It has also been demonstrated that these symptoms can be linked to exposure of a naturally occurring form of asbestos known as amphibole[3]. Previous research has shown strong evidence for a link between the type of asbestos a patient is exposed to and the disease state that develops[1,2]. In order to begin the development of therapeutic strategies, a reasonable mechanism of pathogenesis needs to be characterized with supporting evidence.

Mechanisms of fibrotic pathogenesis in other disease states have implicated the involvement of an autoimmune component. Diseases such as scleroderma, interstitial lung disease, and systemic lupus have all shown alterations in the metabolism of collagen producing cells in response to the presence of autoantibodies[4–6]. Specifically, it has been shown that stimulating autoantibodies to platelet derived growth factor receptor (PDGFR) which is a tyrosine receptor kinase (TRK) in fibroblasts, have induced upregulation of collagen 1 in scleroderma [7]. The initiation of PDGFR could provide an explanation for the development of fibrotic tissue in asbestos induced autoimmune disease if the trend holds true.

Murine studies have also shown strong evidence for a relationship between asbestos fiber exposure, antibody production, and collagen expression [8]. In addition, in vitro studies have indicated that mesothelial cells could contribute to the development of fibrosis [9,10]. Serve demonstrated that increased collagen deposition by Met5a cells could be linked to the presence of MCAA[11]. Our study sought to elucidate these results by probing the signaling pathways affected by the presence of MCAA via flow cytometry and ELISA methods.

Materials and Methods

Cell Culture: Non-malignant, transformed human mesothelial cells, Met-5A (ATCC, Manassas, VA) were grown in RPMI medium (CellGro Mediatec, Manassas, VA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and antibiotics. Cells were maintained at 37° C and 5% CO_2 in a humidified incubator.

Human serum samples: Serum samples were collected by the Center for Asbestos Related Disease (CARD) in Libby, Montana in accordance with Idaho State University IRB project approval #3292MOD and was stored at -80°C until needed. Samples previously identified as MCAA positive (+ive) or negative (-ive) were pooled (Marchand et al., 2012) and small aliquots stored at 20° C. Sera cleared of IgG antibodies were used as negative controls. Sera were cleared using Protein A Agarose Beads (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. IgG removal was confirmed by running sera on a 12% Bis–Tris SDS-PAGE gel (Novex, Life Technologies, Carlsbad, CA), staining

with GelCode Blue Stain Reagent (Thermo Scientific) and checking for lack of bands corresponding to the molecular weight of IgG heavy and light chains (50 and 25 kDa).

Signal induction and fixation: Met5a cells were plated into 6 well plates at 1 million cells per well and the plates were incubated 3 hours to allow the cells to adhere. Afterwards, the wells were treated according to their treatment group designation. After slight agitation insured even distribution of the serum components, the cells were incubated at 37° C for 45 minutes. The cells were then rinsed with cold PBS, scraped up and centrifuged at 4° C for 2 minutes. The supernatant was aspirated. The pellets were then resuspended in fixation buffer for 20 minutes. The cells were washed 2X in Perm/Wash buffer (eBioscience, San Diego, CA) by centrifugation followed by resuspension. The pellets were finally resuspended in 50 uL of Perm/Wash Buffer

Staining and Flow cytometry analysis: After blocking in 3% BSA/PBS, the permeabilized cells were stained with a mouse IgG anti-phosphotyrosine antibody (Santa Cruz Biotech, Dallas, TX) overnight. 400 uL of permeablization buffer was added to the reactions. After 2-3 minutes, the tubes were centrifuged and the buffer aspirated off, these two steps were repeated a second time. A secondary blocker containing an anti-mouse IgM (Santa Cruz) blocker at 1/1000 dilution was added and incubated 15 minutes at room temperature or at 4^{o}C overnight. This was removed using the same washing steps. A secondary autoantibody was added using anti-mouse IgG conjugated to AlexaFluor 488

(Life Technologies/Fisher Scientific, Grand Island, NY). This was washed using the same wash steps. After the final centrifugation, the pellets were resuspended in 500 uL PBS and transferred to flow cytometry tubes. The samples were analyzed using a FACS Calibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA). Mean fluorescence intensity of each sample group was used to compare the effects of the various conditions. Staining with secondary antibody only was used for background staining.

Collagen Deposition via ELISA: Human Met5a cells were plated into a 96 well plate at 70,000 cells per well. The plates were incubated at 37°C for 3 hours. The cells were then treated according to their treatment group designation with an n=6. The cells were then incubated for 82 hours at 37° and then rinsed once with PBS. All blocking and incubation steps were performed under agitation at room temperature for 1 hour with washing steps occurring immediately following incubation steps. The wells were then blocked with 200 uL 5% non-fat dry milk in PBS. The wells were incubated with 2 ug of mouse IgG targeted to collagen type 1 (Abcam, Cambridge, MA) in 100 uL of 3% BSA. The plates were then washed 3X with 200 uL of 0.05% Tween in PBS for 4 minutes each wash. The plates were then blocked again with 5% Milk in PBS. The plates were then incubated with goat anti-mouse IgG conjugated to HRP (Life Technologies). The plates were then washed 3X with 200 uL of 0.05% Tween in PBS. The plates were then developed with 100 uL of Pierce One Step TMB ELISA reagent. The reactions were stopped with 50 uL of 1M HCL. The plates were then read for absorbance at 450 nm on a plate reader (BioTek Instruments, Winooski, VT).

Statistical analyses: One way ANOVA or 2-tailed t-tests were performed using SigmaPlot version v5 (StatPlus Software, Walnut CA). Statistical significance was defined as P values <0.05. Data are graphed with error bars indicating standard error of the mean (SEM). All graphs are representative of at least 2 experiments.

Results

We compared the profiles of phosphorylation between populations of cells exposed to MCAA-positive serum, MCAA-positive serum cleared of all IgG (Cleared), and unexposed cells. We observed that tyrosine phosphorylation was upregulated in cells that had been exposed to MCAA+ serum, but not cleared serum (Figure 3.1 A). Statistically significant serine/threonine phosphorylation was not observed (data not shown). This indicated that differences in tyrosine phosphorylation-mediated signaling were more likely to contribute to the observed collagen deposition than pathways mediated by serine phosphorylation. We also were able show that tyrosine phosphorylation increased in a dose dependent manner in response to increased concentrations of MCAA+ serum (Figure 3.1B).

When we examined the early stages of cell signaling with these same treatment groups, the levels of mean intensity observed in cells pre-treated with Genistein, a tyrosine kinase inhibitor, staining for phosphotyrosine matched the levels of phosphorylation in cells that had not been treated (Figure 3.2), confirming that pathways involving tyrosine phosphorylation were being triggered in response to MCAA exposure.

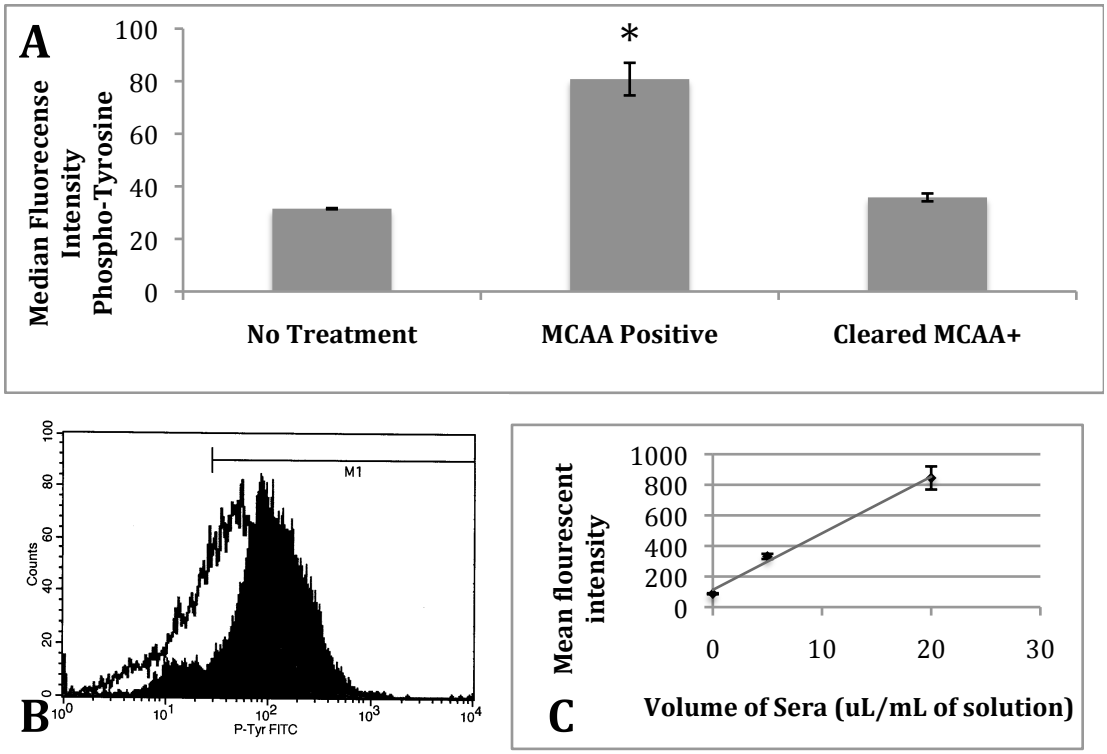

Figure 3.1. Tyrosine phosphorylation in Met5a cells exposed to MCAA via Flow Cytometry flourescence: Met5a cells were exposed to MCAA+ serum for 45 minutes. Populations exposed to serum cleared of IgG and a baseline control were also prepared. The cells were fixed, permeabilized, and stained for tyrosine phosphorylation. **A)** Shows the Median fluorescence intensity of the three treatment groups. **B)** Shows the peak shift between cleared serum(open peak) and MCAA+ serum (solid black) **C)** The mean fluorescensce of cells stained for phosphorylated tyrosine residues was measured against the volume of MCAA positive serum to which the cells had been exposed. The observations from the samples were analyzed using a linear regression model via StatPlus[12]. Both the slope and the intercept were found to be significant ($p<0.05$) with an r^2 of 0.95.

When Genistein was added prior to MCAA exposure to inhibit tyrosine phosphorylation in cells, it was observed that collagen deposition three days later was reduced to nearly the same level observed in cells exposed to serum cleared of IgG (Figure 3.3). This suggests that the upregulation of tyrosine phosphorylation was linked to signaling pathways that contribute to the excessive collagen deposition observed in the cultures.

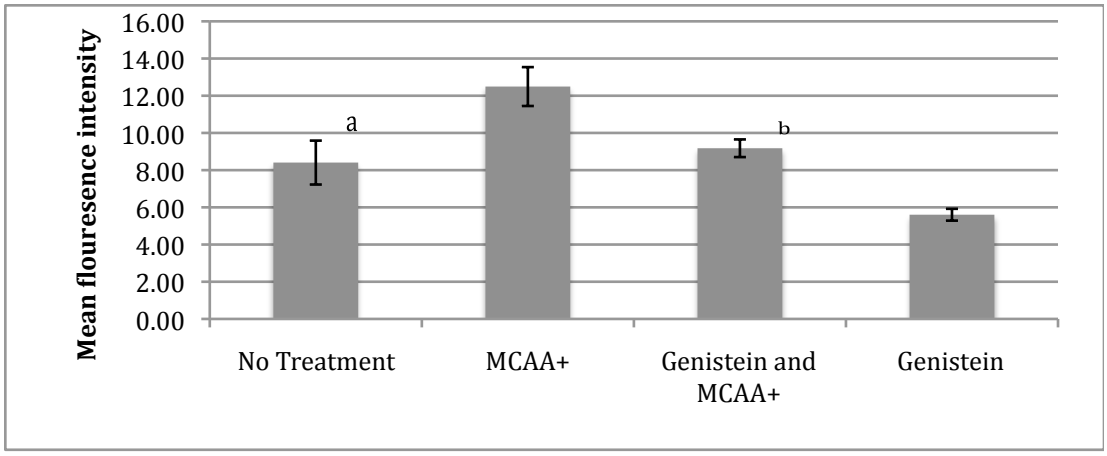

Figure 3.2 Phosphorylation inhibition: The overall phosphorylation of tyrosine residues in untreated cells was compared to those of Met5a cells exposed to MCAA+ serum and Met5a cells exposed to Genistein prior to MCAA+ exposure. Results were found to be significant via a one way ANOVA ($p<.05$). 2-tailed t-test indicates MCAA+ serum significantly increased tyrosine phosphorylation compared to no treatment (a) that was significantly inhibited by Genistein (b).

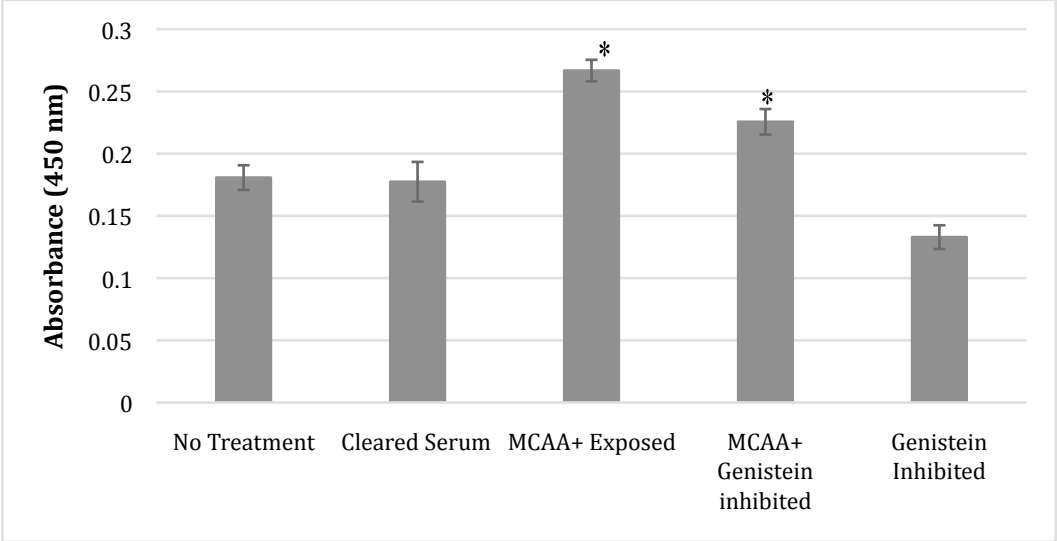

Figure 3.3 Collagen Depostion by Met5a cells: The mean collagen deposition of Met5a cells inhibited with Genistein and exposed to MCAA+ serum was compared to the collagen deposition of untreated cells (No Treatment) and cells exposed to cleared serum (Cleared Serum). Uninhibited cells exposed to MCAA+ serum served as a positive control. Cells exposed to only Genistein were used to account for Genistein's effect on cells under conditions without MCAA+ serum. The error bars shown indicate standard error of the mean. It was found that differences between MCAA+ exposed cells and those exposed to MCAA+ and MCAA+ plus Genistein were significant via a one way ANOVA ($p<0.05$). * = $P < 0.05$ compared to both No Treatment and Cleared Serum.

Discussion

Autoimmune components have been implicated in many forms of fibrosis and other systemic diseases[7,12]. It can be seen from these data that the presence of MCAA results in the upregulation of overall tyrosine phosphorylation. We have also shown that excessive collagen deposition is reduced when tyrosine phosphorylation is pharmaceutically inhibited.

One limitation to this study is the existence of multiple TRK linked to the regulation of ECM and fibrosis[7,13–15]. This creates a situation where tyrosine phosphorylation upregulation could be both a cause and a response of excessive collagen deposition. While further research needs to be done to clarify this issue, it is possible to infer some possibilities from the existing data.

The data that collagen deposition was only partially reduced by TRK inhibition with the addition of Genistein suggests that the two possibilities do not need to be considered as mutually exclusive. It is also important to note that the flow cytometry examination of phosphorylation was performed within the first hour of induction. This means that the cells would not have had time to create the sufficient excess of collagen necessary to attribute the upregulation of tyrosine phosphorylation to increased interactions between collagen and collagen receptors located on the cellular surface. This suggests that the tyrosine phosphorylation was part of pathways involved in upregulating collagen matrix formation.

Alternatively, the partial reduction of collagen deposition might be explained by degradation of Genistein, which occurs at a faster rate than the

degradation of MCAA. Genistein has a half life of 3.2 hours at 37°C[16]. It has been demonstrated that some forms of IgG can exist up to a month[17,18]. It is therefore possible that MCAA induction of tyrosine signaling was only delayed rather than completely halted. Whatever the case, these data clearly support the idea that excessive collagen deposition is, in part, the result of an upregulation in tyrosine phosphorylation pathways.

Does fibrosis occur because of increases in collagen expression or decreases in the degradation of collagen? This is a crucial question that has yet to be conclusively answered. Serve showed that MCAA did not induce epithelial phenotype changes, as has been seen in other fibrotic autoimmune diseases[9,11]. This means that certain aspects of the LA LPT are unique. It was also shown that when collagenase MMP8 was inhibited, collagen deposition was reduced [11]. Our work in Chapter 2 indicated that when plasminogen, and by extension MMP activation, is inhibited, collagen deposition increases. It is clear that further experimentation is required to resolve these differences in results.

Conclusions

In this study, we have demonstrated that the overall phosphorylation of tyrosine residues in Met5A cells exposed to MCAA was statistically different than levels found in unexposed cells. We have also shown that excessive collagen deposition is reduced when tyrosine phosphorylation is inhibited. This indicates that some forms of MCAA are likely to induce the upregulation of signaling pathways that include tyrosine phosphorylation, and this results in the increased formation of collagen matrix. This information is necessary for a

complete understanding of the mechanisms of excessive collagen deposition induced by MCAA and can contribute to the general understanding of LPT and fibrotic diseases.

Future Work

To further clarify these results, we recommend that a tyrosine phosphorylation array be used to assess the specific TRK mediated signaling pathways that are being upregulated in cells exposed to MCAA. This will further clarify and confirm whether or not the observed collagen deposition is the result of a dysregulation in the production or degradation of collagen. If the results indicate that a pathway involving a surface protein such as PDGFR was being activated, then it would be a simple matter of confirming its involvement in the increased collagen deposition. This could be done using an inhibitor that specifically targets PDGFR.

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Chapter 4

Conclusions

Discussion/Conclusions

Approximately 2 million metric tons of asbestos was mined worldwide in 2014. Approximately 50% of this total was mined in Russia, and about 20% was mined in China. A significant portion of this asbestos is shipped to other countries and used in building material(Robert L. Virta, 2015). Asbestos exposure and its consequences of malignant and fibrotic diseases is a current and extensive public health problem. Although mining of asbestos is banned in the U.S., mining of vermiculite is not. The vermiculite mine in Libby operated from 1916 to 1990, and from 1963 on, shipped asbestos contaminated vermiculite to over 200 processing sites in the U.S. (Whitehouse, 2004; Wright et al., 2002). Add to this the exposures that will occur as naturally occurring deposits of asbestos are disturbed through expanding land use in areas like Nevada's southern region (Buck et al., 2013). Widespread asbestos exposure has already happened, and it will still take many years to deal with the consequences and find suitable alternatives. It has also been shown that new technologies, such as carbon nanotubes, are creating materials that lead to similar fibrotic effects(van den Brule et al., 2014; Chen et al., 2014; Glista-Baker et al., 2012). The importance of the overall effort to understand fibrotic diseases and identify relevant targets for therapeutic strategies cannot be overstated. This work provides a good foundation for work that should provide even more understanding about LPT.

When we began this endeavor, we expected to observe binding of MCAA autoantibodies to PDGFR, or some other TRK. The results have yet to disprove

that hypothesis completely, since we are uncertain of our method's ability to capture all of the integral membrane proteins. In addition, finding that plasminogen was one of the target proteins has expanded our view of the systems that could be involved in the development of fibrosis. It had always been a possibility that MCAA were composed of a collection of antibodies that targeted various proteins on the cellular surface. The results of this study indicate that this is the most likely situation.

We have shown that tyrosine phosphorylation is clearly upregulated in cells exposed to MCAA positive serum and that this effect can be halted using a tyrosine kinase specific inhibitor. To be relevant, this work will require further investigation into which specific pathways were upregulated in comparison to the pathways upregulated by exposure to serum containing no MCAA. That work is underway in our laboratory.

The future work that needs to be done to follow up on these findings is very simple. Initially, The role of plasminogen in the excess collagen deposition as it has been observed in a murine model will need to be confirmed. Since maintenance of the ECM involves many regulatory systems, it is important to confirm that the manipulation of one system is enough to lead to the observed symptoms. If the collagen deposition can be affected, then we have truly found at least one target for MCAA, and if not then further testing of other target candidates will be needed.

These findings are critical to the overall objective of finding therapeutic approaches to the unique and devastating pleural fibrotic disease caused by exposure to LA and exacerbated by autoantibodies. Fibrosis is notoriously difficult to treat due to our limited understanding of the underlying, and highly complicated, mechanisms related to scar tissue formation. While the body must form scar tissue in order to heal, this process absolutely must be controlled to prevent excessive and/or progressive collagen deposition. Our findings put us at the cutting edge of understanding the combined roles of matrix deposition and matrix remodeling that must be taken into account. It is our hope that in the near future, we will be able to test specific inhibitors in our animal models to prevent asbestos-induced pleural disease.

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