Macrophage and Fibroblast Interaction in the Setting of Idiopathic Pulmonary Fibrosis

Undergraduate Research Thesis

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by

Shruthi Sethuraman

The Ohio State University

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Project Advisor: Megan Ballinger, Department of Internal Medicine

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a fatal disease characterized by excessive collagen deposition in the lungs which can lead to death caused by respiratory failure. There is no cure for IPF, and the average life span after diagnosis is 3-5 years. Fibroblasts, the main effector cell of pulmonary fibrosis, are responsible for producing the excessive collagen deposition in the lung. Recent work has described an important role for macrophages in regulating the development and propagation of pulmonary fibrosis. Macrophages are circulating innate immune cells whose main functions are to take up pathogens or debris and secrete cytokines or chemokines. Recent work has demonstrated that macrophages isolated from IPF patients are alternatively activated and express different genes when compared to macrophages isolated from normal, donor controls. The purpose of this study is to examine crosstalk between fibroblasts and macrophages and elucidate the mechanism by which macrophages regulate collagen expression in fibroblasts.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disorder characterized by lung injury, fibroblast hyperplasia, and deposition of extracellular matrix that results in reduced lung elasticity, impaired gas exchange and decreased pulmonary function. While not all of the risk factors for developing IPF are known, previous studies have determined that male smokers over the age of 65 are most at risk (1). IPF is deadly with a survival of only 2-5 years following diagnosis (2). Currently the only cure for people suffering from IPF is lung transplantation, which is problematic because many of the IPF patients do not meet the metrics for transplantation. One recent advancement in the field of pulmonary fibrosis has been the production of two antifibrotic therapies, Pirfenidone and Nintedanib, and while these drugs cannot reverse lung damage, they slow the rate of progression of the disease and offer patients more time (3). One of the main challenges surrounding IPF is the lack of clarity regarding disease diagnosis. A multidisciplinary approach for diagnosis of IPF involves clinicians, pathologists, and radiologists is necessary to demonstrate an “usual interstitial pneumonia” on radiography or biopsy with the presence of honeycomb cysts, in addition to ruling out all other interstitial lung diseases (4).

Fibroblasts, the main effector cell of pulmonary fibrosis, are responsible for secreting extracellular matrix (ECM), which is rich in a variety of different proteins including fibronectin, proteoglycans, and elastin, and type I and type III collagen. Under homeostatic conditions, fibroblasts are quiescent cells; however, when the alveolar epithelial cells are injured and the fibroblasts become exposed, a protective response is initiated to repair the wound. This repair response consists of fibroblast activation and cell proliferation and is crucial in wound healing.

Collagen is the most abundant protein in the human body and provides important structure to a variety of different organs by forming fibrils in a triple helix format which further condense into fibers and networks (5). Although there are many different types of collagen, the

different subtypes vary in formation of the helix structure. During a wound response, fibroblasts migrate and proliferate to the site of the wound and secrete ECM proteins to provide a scaffold for new tissue growth (5). While this function is important for injury repair, the issue arises when this process becomes dysregulated resulting in excessive collagen deposition. In the context of IPF, the elevated amounts of collagen in the lungs reduce lung elasticity and impair gas exchange. This excessive collagen deposition is observed via CT scan as a “honeycombing” pattern. While fibroblasts play a role in the propagation of fibrosis, it is clear that there are other interactions between structural and immunological cells that are also important for the development of IPF.

Recent work has described an important role for macrophages in regulating the development and propagation of pulmonary fibrosis. These innate immune cells phagocytize bacteria, debris, and parasites and produce immune effector molecules, such as cytokines and chemokines, to recruit and activate other cells to aid in the immune response. Unlike other immune cells, macrophages activation is a not a result of terminal differentiation, but rather these cells are flexible and capable of responding to their environmental milieu. Previous work has established a variety of different stimuli that can regulation the activation and differentiation phenotype of macrophages (6). For instances, when exposed to IFN-γ and/or LPS macrophages become classically activated and can eliminate microbes through the production of NO and reactive oxygen species. On the other hand, if macrophages are exposed to IL-4 and/or IL-13, they will become alternatively activated, which are important in allergic and wound healing responses (6). Previous work has demonstrated that macrophages isolated from both murine models of pulmonary fibrosis as well as cells from IPF patients have a profibrotic macrophage phenotype (7). Our lab has classified macrophages isolated from a murine model of pulmonary fibrosis to be profibrotic based on increased production of IL-13 and TGF-β, elevated expression of alternative activation markers (such as arginase and Fizz1) and

increased STAT6 phosphorylation (8). These profibrotic macrophages secrete increased cellular mediators which can affect other cells in the lung and propagate the fibrotic response.

The ability of macrophages to respond to signals in the environment is mediated in part by pattern recognition receptors, such as Toll-like receptors (TLRs). Previous work has demonstrated that there are 12 TLRs identified in mouse and 10 in humans these receptors expressed both extracellular and intracellularly identify conversed motifs present in a variety of pathogens, including viruses, fungus, bacteria, and parasites (9). In addition to recognizing pathogen associate molecular patterns (PAMPs), TLRs have also been shown to play an important role in recognizing damage associated molecular patterns (DAMPs), which are released in setting of non-infectious injury and inflammation. Although Toll-like receptors (TLRs) are a major class of PRRs, there are several other classes of PRRs, including C-type lectins, RIG-I helicases (RLH) and nucleotide binding domain and leucine-rich repeat containing receptors [NOD-like receptors (NLRs)] which also play an important role in protecting the host from a myriad of pathogens and danger molecules (9)Most TLRs have been shown to activate the immune system, by recognizing various epitopes and conserved molecules in the environment and recruiting immune cells to the site of invasion. The diversity of different TLRs provides the immunological system of the host with a distinct advantage against a variety of invading pathogens and dangerous molecules. In the scope of IPF, previous work has shown that there is an upregulation of TLRs 2, 3, 4, and 9 in IPF patients (10). However, the implication of altered TLR expression and how this regulates macrophage activation in the setting of IPF has yet to be determined.

In order to advance the current knowledge, our work has focused on understanding cell-to-cell interactions that occur between macrophages and fibroblasts in the setting of pulmonary fibrosis. Previous work done in renal interstitial fibrosis has demonstrated a strong link between renal fibroblasts and renal macrophages in the development of fibrosis (11). Deletion of specific

markers, such as allograft inflammatory factor-1, on the surface of macrophages inhibited the profibrotic phenotype and reduced fibrosis in a human renal fibroblast model (11). In addition, previous research has shown in fibrosis caused by traumatic injury, and that macrophages are necessary, for normal wound healing to occur (12). This work demonstrated that classically activated macrophages stimulate the immune response by recruiting recruit inflammatory cytokines to the site of the wound, after which monocytes differentiate into alternatively activated macrophages to promote tissue healing and more regeneration. Dysregulation at any stage of this pathway can disruption of the dynamic between the difference macrophage activation phenotypes resulting in enhanced muscle fibrosis (12). The pattern of macrophages having a dominant role in the initiation and development of fibrosis in multiple organ systems demonstrates their important role in regulating diseased phenotypes. Based on these data, we hypothesis that the activation phenotype of macrophages regulates fibroblast activation and collagen production and suggest that a better understanding of these interactions will be essential in the design of novel therapeutics to stop or possibly reverse fibrosis.

METHODS

Cell culture

Normal, donor control lungs, deemed not viable for transplantation, were procured via a contract with LifeLine of Ohio, the organ procurement agency of Central Ohio. Lungs from IPF patients were obtained following IRB approval (2014H0367) from consented patients following lung transplantation. All samples collected from normal and diseased lungs are deidentified and referred to via a code. Resident alveolar macrophages were isolated via ex vivo lavage by instilling ~500 ml of PBS with EDTA into the bronchi and filling each lobe to recover the fluid and cells. AMs from IPF and normal, donor controls were frozen at -80 and then rethawed to be used in co-culture experiments. The viability of the AMs after harvest was approximately 75% based on trypan blue exclusion. Fibroblasts were grown to confluency from disrupted lung tissue for 21-28 days in culture containing RPMI, 10% Penicillin, Streptomycin and Amphotericin B (PSA) and 15% FBS in plastic flasks until confluency and then passaged every 3-4 days. Fibroblasts were maintained in either RPMI media with 10% FBS and 10% PSA, or RPMI with 15% FBS and 10% PSA depending on rate of growth. All cells used for these studies were < 8 passages. For activation assays, fibroblasts were treated with 2ng/mL TGF-β and 10nM PGE_2 for 24 hours before harvesting cells for additional experimentation.

In vitro macrophage-fibroblasts co-culture system

Alveolar macrophages derived from human samples were co-cultured with human fibroblast samples both in a direct contact and transwell system. Fibroblasts were first grown to 80% confluency. Fibroblasts were washed with warm PBS and then serum starved (RPMI, 10% PSA) on plastic 6-well plate for 24 hours. Alveolar macrophages were thawed and measured for viability. Macrophages were washed with 10% PSA media and resuspended in RPMI with 10%

FBS and 10% PSA. Fibroblast media was aspirated after the overnight incubation and then approximately 200,000 macrophages were added to each well. Alternatively, to measure indirect contact macrophages were plated on 0.4 uM culture insert from Fisher Scientific. Cells were allowed to grow overnight for 24 hours and then supernatant and cells were collected for additional measurements.

Quantitative real-time PCR (qPCR):

RNA was extracted from cells or flash frozen lung tissues homogenates by using a Direct-zol RNA miniPrep Plus Kit (Zymo Research) according to the manufacture's instruction. RNA was quantified using a NanoDrop 1000 Spectrophotometer (ThermoFisher). cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher), and gene expression was measured by qPCR using a QuantStudio 3 Real-Time PCR System with 30 cycles using PowerUP SYBR Green Master Mix (Applied Biosystems). Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with GAPDH expression as the endogenous control.

ELISA/ Luminex

Each sample was tested for a variety of cytokines simultaneously by using a Luminex bead method (R&D Systems). Experiments were performed according to manufacturer's instructions and samples were thawed and used directly in the assay which was used to measure levels of IL-1, IL-4, IL-5, IL-6, IL-7, IL-12, IL-13, MCP-1, GMCSF, GCSF, and INFγ.

In order to verify protein expression, additional ELISAs were performed to detect specific proteins in the supernatant. Standards and all reagents used for this experiment were prepared according to manufacturer's instructions on the protocol sheet. The conventional ELISA

procedure was applied to the samples using the R&D Systems ELISA protocol. Samples were read at both 450 and 570nm.

Statistical Analysis

All data are expressed as mean ± SEM and were analyzed using Prism statistical program (GraphPad Software). All data was analyzed using -way ANOVAs and represents a normal distribution.

Results:

Lung tissue and AMs from IPF patients have altered expression of TLR receptors compared to cells and lung tissue isolated from normal, donor controls

Expression of TLRs was measured in whole lung tissue and alveolar macrophages from IPF patients compared to normal donor controls. In all of these experiments, the relative expression of TLRs from the normal, donor controls were averaged together and then relative expression of each of the IPF patients was compared to average expression from the normal patients. Using this method, we there is no difference in the expression of TLRs when examining whole lung tissue from IPF patients compared to normal, donor controls (Figure 1A). When the expression of TLRs was measured in AMs, there was a significant decrease in TLRs 3, 5, 7, and 8 mRNA expression in IPF alveolar macrophages compared to normal donor controls (Figure 1B) and a significant decrease in TLR 7 and 9 expression between WT and IPF whole lung tissue mRNA expression (Figure 1A). These data demonstrate that IPF patients have altered TLR expression in their whole lung tissue, as well as AMs and whole lung tissue when compared similar tissue or cells isolated from normal, donor controls.

Treatment of lung fibroblasts with TGF-β and PGE_2 alters fibrotic genes in normal but not IPF samples

In normal fibroblasts, it is known that TGF-β, a profibrotic cytokine, induces collagen expression and PGE_2, an anti-fibrotic lipid mediator, can inhibit collagen expression in fibroblasts (26). However, in the setting of pulmonary fibrosis, PGE_2 is not able to regulate collagen or cellular proliferation (26). To verify the functionality of our primary human lung fibroblasts collected from normal, donor controls and IPF patients, fibroblasts were stimulated with 2 ng/ml of TGF-ß alone or in combination with an anti-fibrotic mediator 10 nM of PGE_2 for 18 hours. mRNA

expression of Collagen I and Collagen III was measured after harvesting cells. As shown in Figure 2A and 2B, treatment of normal fibroblasts with TGF-β resulted in increased expression of Collagen I and Collagen III. As predicted, PGE_2 significantly inhibited the TGF-β induced collagen production in normal fibroblasts, but not in fibroblasts isolated from IPF patients (Figure 2A and B). These data align well with previously published data which demonstrate an altered phenotype of IPF fibroblasts compared to those isolated from normal, donor controls.

Stimulation of AMs from IPF patients with TLR ligands resulted in elevated expression of pro and anti-inflammatory cytokines

Expression of cytokines and chemokines has been shown to be regulated through TLR-dependent signaling pathways. To determine if altered TLR expression correlates with differential cytokine secretion, AMs from IPF patients and normal donor controls were isolated and stimulated overnight with specific TLR ligands, including LPS (TLR4 agonist), Pam3Cys (TLR 1/2 agonist) and PolyI:C (TLR3 agonist). Initially, expression of a variety of pro and anti-inflammatory cytokines was measured by qPCR. For each of these experiments, the relative expression of the stimulated cells was compared to their unstimulated control and the fold-changes were compiled from n= 5 IPF and n=7 normal donor controls. Stimulation of LPS resulted in increased mRNA expression of TNF-α, IL-1β, IL-10 and IL-13 from both IPF macrophages and normal, donor controls (Figure 3A-D) and there was significantly higher cytokine expression in IPF samples compared to normal donor controls. Stimulation with the TLR1/2 agonist Pam3Cys resulted in increased proinflammatory cytokines (TNF-α and IL-1β) in both normal and IPF macrophages (Figure 3A and B); however, TLR1/2 agonist stimulation only increased IL-13 expression in IPF, but not normal macrophages (Figure 3C). In general, TLR3 stimulation did not result in elevated cytokine production.

To verify cytokine expression, supernatants from these cells were collected and used to determine protein concentration by Luminex and ELISA. In this case, all of the supernatants were run together on the same plate and therefore the unstimulated and stimulated samples were represented on the graphs. From the Luminex experiment, we found significant differences in cytokine production among IPF fibroblasts when they were stimulated with TLR agonists. There was increased expression of IL-6, TNF-α, IL-12, GMCSF, and IL-1β in our IPF fibroblasts when they were stimulated with LPS in comparison to normal unstimulated IPF fibroblasts (Figure 4). In all cases, there was no difference in baseline production of cytokines between normal, donor controls and IPF samples. One difference between the cytokines expression as assessed by qPCR and the protein expression by ELISA was that there was no difference in baseline or stimulated expression of IL-13 between macrophages isolated from normal, donor controls and IPF patients (Figure 5C). Stimulation with LPS resulted in a significant increase in production of TNF-α, IL-10, and IL-1β and in each of these cases, IPF AMs produced significantly more when compared to donor controls (Figure 5A, 5B, and 5D). The addition of Pam3Cys, TLR 1/2 agonist, also induced significant protein levels of TNF-α, IL-10, and IL-1β in IPF samples (Figure 5A, 5B, 5D). These data demonstrate that the altered TLR expression profile in the IPF patients resulted in a skewed cytokine response after in vitro stimulation.

Co-culturing fibroblasts with IPF human alveolar macrophages results in increased Collagen I

In order to understand the role of altered macrophage activation in regulating fibroblast phenotypes, we created an in vitro co-culture system. AMs isolated from IPF patients or from normal donor controls are cultured either directly or on transwell membranes with fibroblasts isolated from normal, donor controls. Expression of collagen was measured in the co-culture settings compared to expression of collagen in fibroblasts alone. When IPF macrophages were cultured directly on top of normal fibroblasts, there was a significant increase of collagen I

expression (Figure 6A). However, co-culture of AMs isolated from normal, donor controls did not result in any changes in collagen I expression (Figure 6A). Interestingly, fibroblasts isolated from the bottom of a transwell membrane with IPF macrophages also had elevated collagen I expression when compared to fibroblasts cultures with AMs isolated from normal, donor controls (Figure 6B). These data demonstrate that IPF macrophage can induce collagen expression from normal fibroblasts, and that this effect is not dependent on cell-to-cell contact.

DISCUSSION

Idiopathic pulmonary fibrosis (IPF) is a fatal disease characterized by excessive collagen deposition in the lungs which can lead to death caused by respiratory failure. There is no cure for IPF, and the average life span after diagnosis is 3-5 years. Fibroblasts, the main effector cell of pulmonary fibrosis, are responsible for producing the excessive collagen deposition in the lung. Recent work has described an important role for macrophages in regulating the development and propagation of pulmonary fibrosis. Our data and others have demonstrated that macrophages from IPF patients have an alternatively activated phenotype which enables them to drive the fibrotic phenotype. The purpose of this study was to examine crosstalk between fibroblasts and macrophages and elucidate the mechanism by which macrophages regulate collagen expression in fibroblasts. Our data demonstrated altered TLR expression in macrophages and whole lung tissue in IPF patients compared to normal donor controls. Stimulation of IPF macrophages with TLR specific ligands, LPS and Pam3Cys, resulted in increased expression of pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines production and expression when compared to normal, donor controls. Alternations in expression of anti-inflammatory cytokines IL-10 and IL-13, were also observed when IPF macrophages were stimulated with various TLR ligands, but the same trends were not observed when examining protein production by ELISA. There was elevated collagen I and III expression in IPF fibroblasts compared to normal donor controls when we treated them with TGF-ß Consistent with previous work, PGE_2 stimulation did not inhibit TGF-β induced collagen in IPF fibroblasts. Using a novel co-culture system, we demonstrated that AMs isolated from IPF patients, but not normal, donor controls, induced elevated collagen expression from normal fibroblasts. Together, these data show that IPF fibroblasts and macrophages display dysregulation on many levels including TLR expression, Collagen I/III expression, and most importantly with each other as shown by the co-culture. We conclude that the diseased

phenotype associated with IPF is directly influenced by macrophages as they are not only involved in increased-inflammatory responses associated with IPF, but they also display a unique pro-fibrotic phenotype which then stimulates fibroblasts to also excessively produce collagen.

Expression of TLRs are important in the scope of IPF because they serve as a signaling mechanism for macrophages to respond to their environment. TLRs allow for communication between macrophages and fibroblasts, which is important in both the initiation and propagation of fibrosis. Altered expression and signaling of macrophage TLRs will provide important information in regard to development of biomarkers of disease or possibly even new therapeutics to target the dysregulated phenotype. Our research has demonstrated important fibroblasts-macrophages interactions which can lead to the development and propagation of the fibrotic response. The ability of macrophages to directly regulate the production of collagen production in our fibroblasts in our co-culture experiment exemplifies the sensitive nature of this interaction and how both contribute to the progression of IPF. Our work has shown that macrophages isolated from IPF patients have a differential expression profile of TLRs which are functional and respond to specific ligand stimulation. In vitro experiments demonstrate that the altered TLRs on the macrophage surface response to ligands and secrete a variety of cytokines which can regulate fibroblast activation and proliferation. This work aligns well with previously published work, which has demonstrated macrophage-fibroblast interaction resulting in enhanced fibrosis in other body organs such as the kidneys and muscle (20, 23). Interestingly, in papers by Raghu and Cholok, they discuss inhibiting TGF-ß as a potential therapeutic option to stall fibrosis as well as recommending that patients take NSAIDS to decrease the inflammatory response (4, 12).

Although our work has provided some important insights into the role of macrophage and fibroblasts using primary human cells isolated from well-characterized IPF patients, there

are some limitations to our currently study. First, all of our IPF samples were collected from explant tissue from patients at the time of transplant, and these samples often only reflect cells from late-stages of the disease, rather than early or mid-stage. This is a major limitation in IPF research in general because it prevents us from gaining a better understanding of what initial symptoms of the disease are, how the disease progresses, and how signaling molecules and markers of disease change over time as fibrosis worsens. Given that all of our samples are derived from transplant patients, it is clear that their condition had deteriorated to the point where their only option was transplantation. In addition, just as we received all of our IPF samples from human transplant patients, we also received all of our normal donor controls from patients whose lungs were rejected as donors for transplantation. This presented a major challenge because even though our normal controls did not have IPF, there was limited data in regard to the patient histories such as smoking, drug history or a major health issues that may have contributed to their death. In addition, given that these lungs were rejected as normal for transplantation, there was usually an underlying issue such as poor oxygenation, for example, that was a confounding factor. Another limitation for our current study is that we were using macrophages outside of their natural environment. The cells were isolated from the lung, but the in vitro stimulation took place in a culture dish, which is radically different from the lung milieu. Previous work has demonstrated that some aspects of macrophage activation and differentiation are epigenetically regulated, which suggests that these changes would be maintained in a culture setting (13, 14).

In the future we hope to expand this research by continuing to examine the relationship between macrophages and fibroblasts. We would like to expand our current work by treating IPF and normal lung fibroblasts with a variety of different TLR agonists to study stimulation with specific ligands and regulate cytokine production as well as expression of collagen and other ECM proteins. We also would like to measure chemokine and cytokine receptors on fibroblasts

to identify if there are any differences in expression of these receptors on IPF fibroblasts compared to normal, donor controls. This information would be important in understanding how cytokine and chemokines can regulate fibroblast activation. Additionally, we acknowledge that in vitro conditions can greatly affect the activation and gene expression profiles of our cells. In order to more accurately reflect the lung microenvironment, we have begun culturing lung fibroblasts on soft, gel coated plates and will assess their ability to produce ECM proteins using our co-culture system.

While the novelty of this study is the use of human samples, this is complicated by the heterogeneity of the disease and difficulty in diagnosing IPF patients. There are many different interstitial lung diseases (ILD), but IPF has the most severe mortality; therefore, there is still a need to better define risks of disease such as exposures, to chemicals or toxins at their work, as well as genetic markers and aging associations to help further classify this sporadic and deadly disease (15). Attempts have been made to try and rectify this; however, it is still a problem and according to the European IPF Registry, over half of the patients initially inputted in the registry actually had interstitial lung disease, and not IPF (16). With such similar symptoms and disease presentations, it is obvious that distinguishing between ILDs and IPF is a crucial task for pulmonologists. Among the population that was determined to have IPF, physicians also saw the presence of many co-morbidities in IPF patients such as arterial hypertension and GERD. Likewise, there is a subpopulation of IPF patients who may display relatively stable health, but may suffer a precipitous decline in health, termed an acute exacerbation, in association with an infectious event, most likely a viral pneumonia (17). Our work provides the important framework for using AMs as a possible biomarker of IPF. We demonstrated an altered TLR receptor profile expression from AMs isolated from IPF patients. This cellular population, which is relatively easy to access to access in human patients via a procedure called a bronchoalveolar lavage and could provide essential information regarding the immunological environment in the lung.

While the development of therapeutics for IPF marked immense progress, there is still a lot of work to be done in identifying more specific markers of IPF as well as understanding the mechanism by which fibroblasts produce excessive collagen deposition. Our work suggests that the interactions between macrophages and fibroblasts provides a unique system in which to identify novel biomarkers or signaling molecules important in preventing IPF and possibly in reversing its effects.

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