Translational Science

Sarcoidosis, alveolar β-actin and pulmonary fibrosis

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Summary

Background: Sarcoidosis is a multisystem granulomatous disease of unknown aetiology. Proteins present within the alveolar space early in sarcoidosis disease may provide an insight into novel mechanisms for the development of fibrotic disease and in particular pulmonary fibrosis. Methods: A modified two-dimensional difference gel electrophoresis protocol was applied to the human bronchoalveolar lavage fluid (hBALF) of four patients with non-persistent pulmonary interstitial disease at 4-year follow-up (defined as mild disease) and four patients who developed pulmonary interstitial disease at 4-year follow-up (defined as severe disease). The protein β-actin was identified by LC-MS/MS from a preparative gel and found to be significantly elevated in early lavages from the severe disease group. To look at the potential pro-fibrotic effects of this protein, primary human pulmonary fibroblasts (CCD-19Lu) were treated with recombinant β-actin following which qPCR and ELISA assays were used to measure any effects.

Results: We found that β-actin levels were significantly elevated in early hBALF samples in patients who subsequently developed severe disease when compared to the mild group. Treating primary human pulmonary fibroblasts with recombinant β-actin led to enhanced gene expression of the pro-fibrotic markers alpha smooth muscle actin and collagen 1 as well as the increased secretion of interleukin-13 and metalloproteinases 3 and 9.

Conclusion: Free β-actin within the lungs of sarcoidosis patients potentially may contribute to disease pathogenesis particularly in the context of abnormal remodelling and the development of pulmonary fibrosis.

Introduction

Pulmonary fibrosis, no matter what the cause, remains a scientific enigma where the precise mechanisms driving this pathogenic progressive process are not clearly understood.1–4 In the context of sarcoidosis the natural history of patients is highly variable; spontaneous remission of the granulomatous inflammation occurs in the majority of patients, but up to 30% of patients can develop persistent disease.5 Ireland has one of the highest prevalences of this disease with up to 85 cases per 100 000 reported.6 In this study, we wished to utilize a proteomics approach to interrogate bronchoalveolar...
lavage samples to detect differential protein expression in initial samples derived from patients who subsequently resolved compared to patients whose disease ultimately progressed. It was our unifying hypothesis that specific novel proteins would be detected that had the capacity to contribute to aberrant remodelling and repair leading to progressive pulmonary fibrosis in sarcoidosis.

In our preliminary study we found that elevated β-actin in the alveolar fluid of patients with sarcoidosis at initial diagnosis significantly correlated to the subsequent development of progressive pulmonary fibrosis requiring immunosuppressive therapy within 2 years of follow-up. To investigate whether ‘free’ β-actin may contribute to aberrant remodelling and repair, we then looked at the capacity of β-actin to induce an enhanced pro-fibrotic phenotype on naïve primary human pulmonary fibroblasts.

**Materials and methods**

Recombinant human β-actin (orb84841) was purchased from Biorbyt, UK. Cytokine ELISA assays were obtained from meso scale discovery, USA [K151ASB-1 Human Interleukin-13 (IL-13) Tissue Culture Kit, K15010B-1 Human TH1/TH2 10-Plex Tissue Culture Kit, K151UC-1 Human TGF-β 1 single plex ELISA Kit, Human MMP-3 (matrix metalloproteinase-3) plex (MMP-1, 3, 9) K15034C-2]. Antibodies for western blot analysis were purchased from Sigma-Aldrich (A3853), Ireland and Abcam, UK. CCD-19Lu primary pulmonary human fibroblasts were purchased from the ATCC culture collection (ATCC Number: CCL-210). Human bronchoalveolar lavage fluid (hBALF) from mild and severe sarcoidosis patients was initially depleted on the SepproTM HSA-IgG liquid chromatography column before being separated on two-dimensional (2D) difference gel electrophoresis (12% homogenous SDS-PAGE). Progenesis SameSpots software analysis was used to identify the spots from the hBALF that differed significantly (t-test) between mild and severe sarcoid patients.

qPCR was performed on the Agilent/Stratagene MX3000P system using the following primer sequences: hz-SMA forward CGTTACTACTTGAGGAGCA, reverse AACGTTCTATTTCCGATGGTG; h β-actin forward GGACCTCGAGGAAGATGG, reverse AGCACTGTGGTGCGTCAGAC; B2M forward AGGCTATCCAGCGTACTCCA, reverse CCA GTCCTGTGTAAGACA; collagen type I alpha 1 forward GAACCGGTGTACCTCCGT, reverse GA ACGAGGTAGTCTTTCA.

**Cell-culture**

CCD-19Lu cells were maintained in Eagle’s minimum essential medium with 10% FCS, pen/strep antibiotics. Medium was changed twice weekly and cells were split 1:4 when they reached 80–90% confluence. For β-actin treatment 2 × 10^5 cells were seeded in six-well plates overnight before being treated with 1 g/ml β-actin and left for 24 and 48 h. After this supernatants from the cultured cells were removed and stored at −20°C for later cytokine analysis and cells were washed in PBS before the addition of trizol for RNA extraction and gene expression analysis by qPCR. ELISA assays were performed using the supernatants from the cell culture experiment according to their manufacturer’s instructions.

**Western blots**

One-ml volumes of hBALF were assessed for total protein levels using a BCA assay for normalization prior to loading on to a 12% SDS-PAGE gel. Following electrophoresis gels were analysed for the presence of proteins using Ponceau S solution before overnight transfer to a nitro-cellulose membrane. Membranes were then probed for human β-actin and GAPDH (as a loading control).

**Bronchoalveolar lavages**

All BALs for the study were carried out by the same operator and standardized under the published guidelines of the ATS.7

**Study population**

Our hypothesis was tested using a 4 × 4 comparison study design (n=8) of sarcoidosis patients who presented to St Vincent’s University Hospital, Dublin, Ireland and were evaluated and subsequently managed by a single physician (S.C.D.). All were Caucasian, non-smokers and had a tissue proven diagnosis of sarcoidosis. All patients had follow-up data collected for at least 4 years post-diagnosis. Patients were selected based on four who had self-remitting pulmonary disease at 2-year follow-up (defined as mild) vs. four patients who had persistent progressive pulmonary disease and/or required corticosteroid therapy, over a 2-year follow-up period (defined as severe). This classification of either persistent or non-persistent disease was based on a modification of a system previously described.8 Briefly, persistent disease was defined as those patients at 2-year follow-up who had (i) chest radiograph stage II or stage III with associated abnormal pulmonary function parameters FVC and or total lung capacity and or a transfer factor <80%
predicted values or (ii) chest radiograph stage IV or (iii) prescribed corticosteroids over the 2-year follow-up period for persistent pulmonary disease. Corticosteroids were prescribed in patients exhibiting a significant deterioration in pulmonary physiological parameters (DLCO decrease $>15\%$) associated with radiographic deterioration. Corticosteroids were also prescribed as per international guidelines. No patient had their BALF included in other proteomic experiments. St Vincent’s University Hospital Medical Ethics committee approved the study. The four mild patients were two male two female, presenting with BHLA (bilateral hilar lymphadenopathy), no erythema nodosum with clinical/radiological and biochemical resolution of disease within 2 years of diagnosis. No patient required treatment. The severe patients were two male, two female patients presenting with BHLA, no erythema nodosum who developed symptomatic and pulmonary radiological deterioration requiring corticosteroid medication within 2 years of diagnosis. All eight patients had biopsy proven disease. All patients were non-smokers. All patients were Silzbach stage 1 (BHLA) at initial clinical presentation. All BAL procedures were performed at initial clinical presentation/diagnosis. None had evidence of extrapulmonary disease at initial presentation. A student t-test was used to analyse the results obtained for significance and where data failed normality testing (Shapiro–Wilk analysis) a Mann–Whitney rank sum test was performed.

**Results**

β-Actin is a highly conserved and intrinsic protein that has major roles in cell motility, structure and integrity. Free actin has also been shown to have a toxicity role in certain diseases.10 We looked at hBALF from four severe sarcoid patients and four mild sarcoid patients. The hBALF was pre-fractionated, thereby reducing the complexity of the proteome. This strategy was designed to enhance overall protein identification across the proteome and specifically to improve detection of low molecular weight proteins, which typically are of lower abundance. Only albumin and IgG were removed because we wanted to maximize enrichment with as little alteration to the proteome as possible. Topographical maps, generated in Progenesis SameSpots, made up of a composite of mild cases and a composite of severe cases show the differential expression of β-actin and are a visual representation of the fold change. Mass spectrometry was used to identify β-actin (Figure 1). Analysis of hBALF from the severe and mild Sarcoid patients showed that β-actin protein levels were significantly elevated in the severe group with an almost 2-fold increase in expression levels ($P<0.05$). To determine if elevated levels of β-actin had any pro fibrotic effects on human cells, we treated primary lung fibroblasts (CCD-19Lu) with recombinant β-actin for 24 and 48 h at a concentration of 1 μg/ml. We found, using the LDH assay, that at higher concentrations of β-actin the protein was toxic to cells (results not shown) hence we used only 1 μg/ml in our experiments. Quantitative PCR analysis of collagen 1 alpha 1 (Col1) and alpha smooth muscle actin (α-SMA) gene expression in response to β-actin showed both genes were significantly elevated after 24 h (Col1 showed a 1.4-fold increase with β-actin treatment vs. control) and 48 h (α-SMA showed a 1.5-fold increase with β-actin treatment vs. control) (Figure 2a and 2b). α-SMA and Col1 expressions were not significant at 24 and 48 h respectively following treatment. Fibroblasts are known to contribute to the deposition of extra cellular matrix proteins such as collagen11 and α-SMA is a common marker for fibroblast/myofibroblast differentiation12–14 and is the cell type most responsible for interstitial matrix accumulation and subsequent structural deformations associated with fibrotic diseases such as sarcoidosis.15

IL-13 pro-inflammatory cytokine was found to be significantly elevated (1.5-fold increase in secretion in supernatants from β-actin treated primary lung fibroblasts when compared with control where $P<0.05$) after 48 h (Figure 3a). We also found elevated levels of both MMP-3 ($P<0.05$) where there was a 1.5-fold increase in secretion of the protein from the treated primary lung fibroblasts when compared with untreated after 48 h (Figure 3b). While MMP-9 did not show a significant increase.
in secretion in supernatants from β-actin treated primary lung fibroblasts (Figure 3c) when compared to control it was still elevated (a 1.4-fold increase in MMP-9 was observed in supernatants from treated cells when compared to control).

Analysis of total and active TGF-β produced by the CCDs in response to treatment with β-actin showed trends towards increased TGF production after 48 h only but no significant results (data not shown) were observed. IL-8 levels were also significantly elevated at 24 and 48 h (data not shown). IL-5 and TNF-α levels were significantly elevated too after 24 and 48 h but the data showed single digit picogram levels only.

**Discussion**

Sarcoidosis represents a multisystem granulomatous disease of unknown aetiology. While events leading to the formation of non-caseating granulomas seen in this disease are being understood with increasing clarity, the precise mechanisms that either lead to resolution or development of pulmonary fibrosis over time are less clearly understood. It is in this context that we undertook a proteomic screen of BAL samples obtained at diagnosis from two sarcoidosis patient cohorts, those that self resolved and those that developed progressive pulmonary disease over a 2-year follow-up. We found that elevated β-actin in those initial samples correlated significantly to the subsequent development of progressive pulmonary disease and the requirement for immunosuppressive therapy.

The actin family of proteins is highly conserved with multiple intracellular functions ranging from intrinsic mechanical support to cells, to scaffolding for intracellular transport in signalling. Extracellular actin has previously been described as significantly toxic to individual cells. Humans consequently have developed highly efficient *in vivo* actin scavenging systems to limit this toxicity. Systemic extracellular actin has been found previously in acute inflammatory diseases and is felt to be secondary to local tissue injury. In the context of acute inflammation and the development of multiple organ dysfunction syndrome, it has been proposed that extracellular actin contributes towards this injurious inflammatory process when the level of free actin significantly exceeds the protective actin-scavenging systems. In this article, we specifically describe elevated extracellular β-actin in the alveolar space of sarcoidosis patients at diagnosis correlating to subsequent progressive disease.

Historically, β-actin has been viewed as a relatively stable gene in various biological processes and consequently often used as a control gene in assays. In order to address whether our observation simply indirectly reflected local tissue injury or whether free β-actin potentially may have a more pathogenic role in evolving fibrosis, we co-incubated β-actin with primary human pulmonary fibroblasts. We found that ‘free’ extracellular β-actin has the capacity to induce a pro-inflammatory and profibrotic phenotype in human primary fibroblasts. Specifically, we found significant induction of mRNA for the following proteins implicated in the fibrotic response: α-SMA, Col1, IL-13 and MMP-3 and -9. Elevated levels of metalloproteinases have been found in hBALF and recent studies have shown that a MMP-9 polymorphism may predispose a subject to sarcoidosis.

Accelerated α-SMA RNA generation is a marker of myofibroblast generation. This cell represents a key orchestrator of tissue contracture which contributes to excessive tissue injury in progressive pulmonary fibrosis. The myofibroblast has a characteristic phenotype of excessive production of collagenous
extracellular matrix (ECM) such as Col1 and enhanced tensile force.\textsuperscript{20}

IL-13 has been causally linked to the development of fibrosis in a variety of chronic inflammatory disease.\textsuperscript{21} This pro-fibrotic cytokine has generated significant interest within the pharmaceutical industry as a valid therapeutic target in pulmonary fibrosis. Therefore, it is intriguing that extracellular \(\beta\)-actin has the capacity to induce this cytokine which has the capacity to drive a progressive fibrotic response.

MMPs are tightly regulated at the transcriptional and post-transcriptional levels. However, uncontrolled MMP activity results in tissue damage. They have been found to be significantly up regulated in lungs of patients with idiopathic pulmonary fibrosis (IPF) and have been shown to actively participate in the pathogenesis of this disease through ECM remodelling and basement membrane disruption.\textsuperscript{18} MMP-3 has been shown to be up regulated in IPF tissue and MMP-3 knock-out mice are protected from bleomycin induced pulmonary fibrosis \textit{in vivo}.\textsuperscript{22} In addition, the addition of MMP-3 in cellular systems is associated with accelerated epithelial mesenchymal transition (EMT). MMP-9 is significantly elevated in a variety of cells derived from sarcoidosis and IPF.\textsuperscript{23,24} Fibrocytes have generated significant interest as a circulating cell that has the capacity to contribute to the myofibroblast pool within the lung and EMT in fibrotic disease.\textsuperscript{2} MMP-9 is significantly expressed by fibrocytes and this protease has previously been implicated in fibrocyte trafficking into the lung particularly across the basement membrane.\textsuperscript{24}

Our finding that ‘free’ \(\beta\)-actin has the capacity to induce from primary human pulmonary fibroblasts a variety of key mediators implicated in aberrant tissue remodelling and repair supports our hypothesis that \(\beta\)-actin rather than having a neutral bystander role, has the capacity for a more direct pathogenic role in evolving pulmonary fibrosis in sarcoidosis. \(\beta\)-Actin is commonly used as a house keeping gene in qPCR analysis in many lung fibrotic diseases. Our finding would add further support to Kriegova et al.\textsuperscript{25} who previously reported that the use of \(\beta\)-actin as a house keeping gene to normalize

\begin{figure}[h]
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\caption{Pro-inflammatory cytokine IL-13 (a) was found to be significantly elevated (\(P<0.05\)) in supernatants from primary lung fibroblasts treated with 1 \(\mu\)g/ml of \(\beta\)-actin after 48 h. Tissue remodelling proteins MMP-3 and MMP-9 were also found to be elevated (MMP-3 \(P<0.05\)) in supernatants from primary lung fibroblasts treated with 1 \(\mu\)g/ml \(\beta\)-actin after 48 h (b and c). Error bars represent S.E.M.}
\end{figure}
expression profiles in bronchoalveolar cells was not suitable due to the fact that its expression, in comparison to other housekeeping genes, varied considerably between patients.

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**Conflict of interest:** None declared.

**References**


