Biomarkers, 2009; 14(6): 366–371

informa
healthcare

RESEARCH ARTICLE

A novel application for Cocoacrisp protein as a biomarker for experimental pulmonary fibrosis

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Abstract

Pulmonary fibrosis is a debilitating disease affecting up to 2 million people worldwide, with a median survival rate of only 3 years after diagnosis. The aim of this study was to evaluate a potential protein biomarker (Cocoacrisp, CC) to identify the onset of pulmonary fibrosis. A model of fibrosis was induced via intratracheal instillation of bleomycin, and samples were collected during the early phase of the disease. Immunohistochemical identification of CC was carried out in lung tissue from the bleomycin model. Quantification by image analysis showed CC levels were doubled (p <0.0003), after a single bleomycin dose, but not after double instillation. Microscopic analysis revealed that CC signal was primarily detected on the alveolar surface. The secretion of the novel protein CC during the early stages of bleomycin-induced injury may have the potential to be utilized as a clinical biomarker for the early stages of fibrosis, particularly as it may be detectable in bronchoalveolar lavage fluid.

Keywords: *Cocoacrisp; bleomycin; pulmonary toxicity; oedema; secreted protein*

Introduction

Diffuse interstitial lung diseases are part of a very large group of more than 200 different diseases. One of the most common of these diseases appears to be idiopathic pulmonary fibrosis (IPF) (Demedts et al. 2001), also known as cryptogenic fibrosing alveolitis. The exact prevalence and incidence of IPF is not known, but it is thought that the total number of cases worldwide is in the region of 3–30 cases per 100 000, with 5–11 cases per 100 000 diagnosed every year (Fellrath & du Bois 2003). This has doubled in the past decade, and with a median survival of only 3 years after diagnosis, the prognosis is not good (Dempsey 2006). A reliable biomarker for the earliest stages of this disease could speed diagnosis, and improve quality of life and survival rates of patients.

The discovery of novel proteins provides new opportunities for development of drug therapies and biomarkers. In particular, secreted proteins have properties that lend themselves to use as biomarkers (Clark et al. 2003). Their presence in extracellular fluids such as urine, blood or sputum means samples are more accessible, and therefore facilitates monitoring. The cysteine-rich secreted protein (CRISP) family is a large group of secreted proteins with largely unknown function (Smith et al. 2001). One of these proteins is known as Cocoacrisp (CC). The function of CC is still under investigation; however the sequence and basic architecture has been defined (Figure 1). CC contains an SCP module which is found in many species of secretory proteins, including the CRISP family (Ookuma et al. 2003). The main characteristic of this protein is the presence of two LCCL domains. It is thought that the LCCL domain may be involved in the binding of lipopolysaccharide (LPS) (Trexler et al. 2000).

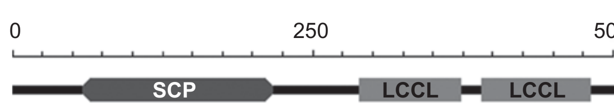

Figure 1. Putative conserved domains for cocoacrisp showing an SCP domain and two LCCL modules. Identified using a Blastp search from NCBI.

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(Received 17 December 2008; revised 08 May 2009; accepted 08 May 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.1080/13547500903029736

One of the most frequently used and best described models of fibrosis is the bleomycin model (Chua et al. 2005). Bleomycins are a family of compounds which show antibiotic and potent antitumour effects. They are widely used in the management of squamous cell carcinomas, malignant lymphomas and testicular teratomas. Unfortunately, the use of bleomycin in the treatment of these diseases is limited by its capacity to cause pulmonary toxicity. Initially bleomycin induces oedema and inflammation, followed by progressive destruction of the normal lung architecture and fibrosis (Shen et al. 1988). In this study, this characteristic was exploited to generate a model of fibrosis. Samples were collected during the early stages and immunohistochemical analysis was used to determine the differential expression of CC in tissue and lavage fluid. It was hoped that this would determine the potential for CC to act as a biomarker for prefibrotic pulmonary injury.

Materials and methods

Experimental model of prefibrotic pulmonary injury

Experiments were performed in accordance with the codes of practice specified by the Home Office (UK), and following approval from the local ethical committee.

Pathogen-free, male Sprague Dawley Rats (Charles River Ltd., Margate, UK) weighing 200–250 g were lightly anaesthetized with halothane (Rhone Merieux, UK) prior to dosing. A singe intratracheal instillation (IT) was given on day 0, followed by a second IT dose on day 7. Animals were treated with 0.5 units of bleomycin sulphate (Kyowa Hakko, UK) suspended in 0.5 ml 0.15 M NaCl ($n=6$). Sham controls received only 0.5 ml 0.15 M NaCl ($n=6$). Animals were closely monitored for the duration of the experiment, using a post-procedure pain and distress score sheet (Wolfensohn & Lloyd 1998). Recovery was rapid with no signs of adverse health effects. Lungs were harvested 3, 7, 10, 14 and 28 days after the initial IT dose.

Immunohistochemistry

After excision from the chest cavity, the lungs were inflated and stored in 10% neutral buffered formalin at 4°C for a minimum of 24 h. After this time the lungs were processed for paraffin embedding and sectioning. Tissue sections (5 μm) were cut using a microtome and antigen unmasking was carried out by boiling in a citric acid-based antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 2 min. This was followed by quenching endogenous peroxidase activity in the tissue by immersing in 0.3% hydrogen peroxide solution for 30 min. Immunostaining was carried out using CC primary antibody (raised in rabbit, in-house). Binding of the antibody was detected by a RTU Vectastain Universal Quick kit (Vector), following the manufacturer's instructions. In brief, a biotinylated secondary antibody was applied, followed by a streptavidin/peroxidase complex. NovaRed (Vector) was used as a substrate for the peroxidase. The sections were then counterstained with light green stain.

Image analysis

Image analysis (IA) was used to capture digitized images of saline- and bleomycin-treated tissue sections. At each time point, light microscope images of random areas over three lung tissue sections, were captured and saved as TIFF files ($n=10$). These images were then imported into the Leica Q550IW Image Analysis System (Leica Microsystems, UK) for image processing and quantification. Initially the white balance was adjusted to normalize the background colour detection levels. The parameters for detection of colour change related to CC were then set. The total area (mm^2) of CC label in bleomycin-treated samples was calculated versus controls.

Statistical analysis

Data comparisons were performed using Student's *t*-test. A *p*-value of ≤ 0.05 was considered significant, and $p \leq 0.001$ was considered highly significant. All data are expressed as mean ± SD, unless otherwise indicated.

Structure and sequence comparison

The protein sequence of human CC (AAK16495) was compared across a large database of proteins using a protein–protein BLAST search (blastp, NCBI). Matches to predicted, hypothetical, or other unnamed CRISP proteins were discarded. The sequences of the remaining significant matches were then compared using DNAsis (for windows) software.

Results

Immunohistochemistry

Microscopic analysis of sections treated with serum control rather than primary antibody was carried out at all time points for both saline- and bleomycin-instilled lung tissue. These samples showed no evidence of labelling (Figure 2D). Equivalent analysis was carried out to determine the expression of CC in bleomycin- and saline-instilled lung. At 3 and 7 days after a single bleomycin dose an increase in CC expression was observed (Figures 2 and 3). CC could be seen mainly lining the

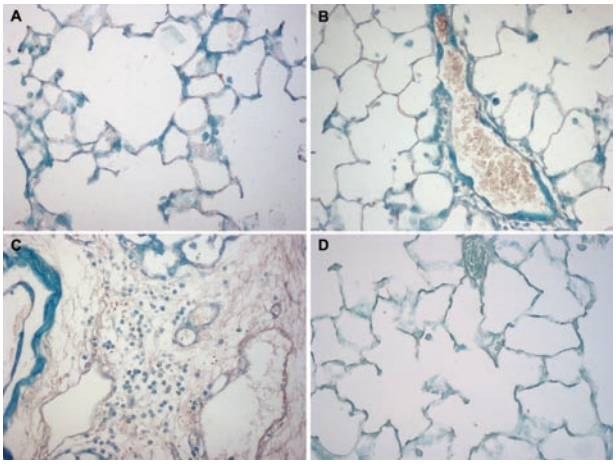

Figure 2. Cocoacrisp expression in sections of lung tissue 3 days after bleomycin treatment (x 200 magnification): (A) alveolar region; (B) blood vessel; (C) region of tissue degradation; (D) serum control stained alveolar region. Cocoacrisp is stained red/brown, $n=6$ (biological replicates).

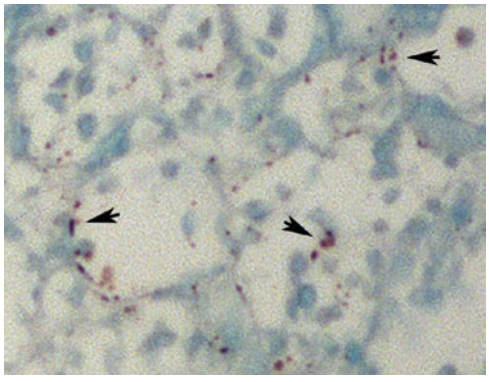

Figure 3. Cocoacrisp expression in the alveolar region of a section of lung tissue 3 days after bleomycin treatment (x 400 magnification). Cocoacrisp is stained red/brown, indicated by arrow heads, $n=6$ (biological replicates).

surface of the tissue (Figures 2A and 3). This occurred primarily in areas of the tissue which appeared more severely effected by the treatment. There was also consistent appearance of CC detected in the blood vessels (Figure 2B). The other significant area of CC expression was in regions of tissue degradation where the lung architecture was largely disorganized (Figure 2C).

Quantification of the images allowed further analysis of CC expression in the tissue. The area (mm^2) of CC label detected in the tissue was used as the value for CC expression. The resulting data are shown in Figure 4. After a single bleomycin instillation the amount of CC expression was more than doubled in comparison to saline instillation. At both 3 and 7 days post-instillation the difference in CC expression in lung tissue was statistically significant with a p-value ≤ 0.0003. After the second instillation, there was no significant difference between saline- and bleomycin-instilled tissue.

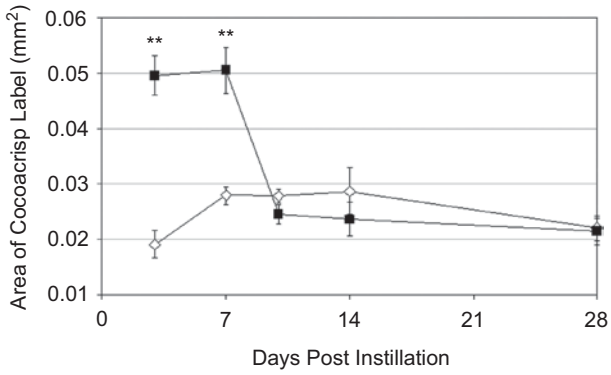

Figure 4. Comparison between the levels of Cocoacrisp expression in bleomycin- and saline-treated samples. Quantification was achieved using the Leica Q550IW image analysis system. **Significant, $p \leq 0.001$.

Structure and sequence comparison

The BLAST search generated a number of full or partial hits; however the majority were either predicted sequences, or other unnamed LCCL domains containing CRISP proteins. These were consequently ignored, which resulted in two significant hits for a trypsin inhibitor (AAQ89150) and late gestation lung protein 1 (Lgl1) (Trexler et al. 2000). These both comprise an SCP sequence and two LCCL domains (Figure 5). Sequence homology was determined as 74% (Lgl1) and 76% (trypsin inhibitor) (Figure 6).

Discussion

This study was carried out to investigate the possibility that CC, a novel secreted protein, might act as an early indicator for the development of pulmonary fibrosis. The technique of bleomycin instillation in rats has been successfully used in the past to generate a model of pulmonary injury (Balharry et al. 2005). It is known to initiate the upregulation of a number of growth factors which may be linked to fibrogenic alterations to the lung.

During this study, in all saline samples there appeared to be a low level of CC expression. This baseline signal appeared to be matched by the samples treated with a double bleomycin instillation. It was considered that some non-specific hybridization may have occurred; however this was negated by the lack of signal in the serum control samples (Figure 2D). CC expression appeared to be significantly elevated 3 and 7 days after a single bleomycin instillation, but as the injury progressed

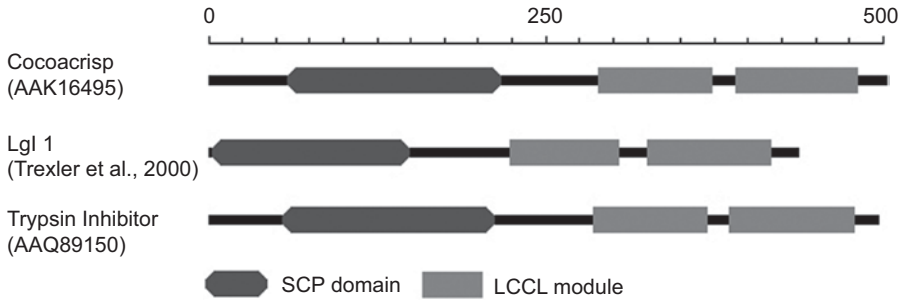

Figure 5. Homology between the conserved domains for Cocoacrisp, late gestation lung protein 1 (Lgl1) and trypsin inhibitor. All three contain an SCP domain and two LCCL modules.

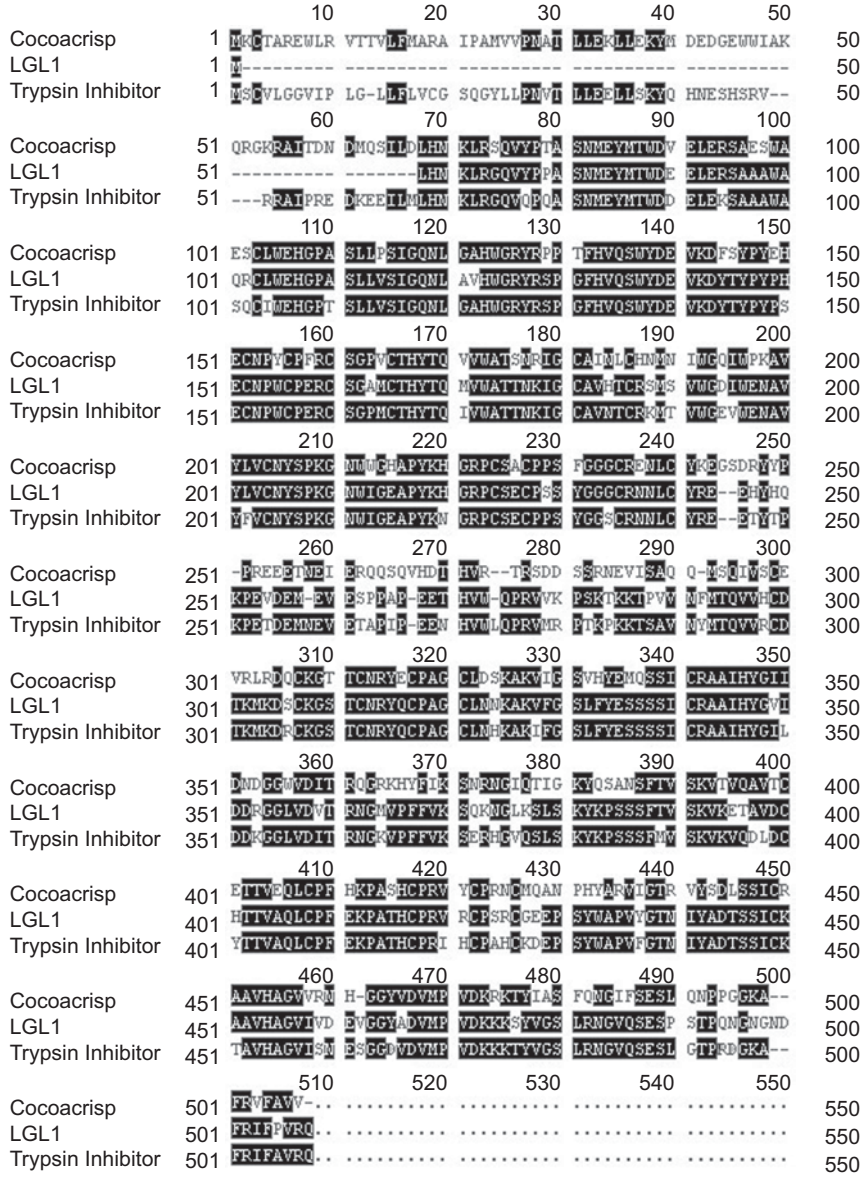

Figure 6. Sequence homology between Cocoacrisp, late gestation lung protein 1 (Lgl1) and trypsin inhibitor.

the amount of CC dropped back down to control levels. Additional dosing with IT bleomycin on day 7 appeared to have no effect on CC expression.

While these elevated levels of CC may be due to region-specific protein expression, the homology of CC to Lgl1, trypsin inhibitor and other CRISP proteins, suggested that it was also a secreted extracellular protein. This appeared to be confirmed by the location of CC on the surface of the lung and in the blood vessels. One of the main characteristics of these proteins is the presence of two LCCL domains. The LCCL domain was first identified in *Limulus* factor C from the horse-shoe crab and the key functional aspect of this protein is its sensitivity to LPS endotoxin. Binding of LPS to factor C initiates a host defence mechanism, protecting the organism from infection (Trexler et al. 2000). Lgl1 protein is involved in lung maturation, and increased expression of Lgl1 has been shown to coincide with the production of surfactant (Kaplan et al. 1999). Similarly to factor C, surfactant proteins SP-A and SP-D bind LPS and therefore play an important role in the innate immune response. The increase in Lgl1 could induce a similar effect as the surfactant components, resulting in protection of the lung from pathogens (Trexler et al. 2000). This indicates that if the similarity in structure between CC and Lgl1 persists to the function of the protein, CC could be involved in the defence of the lung to insult. The appearance of CC on the lung surface would seem to support this theory. If this is the case, it is an immediate, transient response, as the levels rapidly return to baseline levels.

The Lgl1 protein has also been implicated in the regulation of extracellular matrix (ECM) degradation by modulation of trypsin activity (Kaplan et al. 1999). The breakdown of the ECM, particularly proteoglycans, leads to increased vascular permeability (Miserocchi et al. 2001) which is one of the critical events in the development of pulmonary oedema. This damage to the pulmonary epithelium could be sufficient to trigger a fibrotic response (Fellrath & du Bois 2003). Lgl1 causes trypsin inhibition which could result in reduced ECM degradation, and therefore reduced epithelial damage. The other protein with high homology to CC is a trypsin inhibitor (AAQ89150), which would have a similar effect. In fact, antitrypsin activity has previously been shown to mitigate fibrosis induced by bleomycin administration (Nagai et al. 1992). Again, assuming a similar role for CC as Lgl1 and the trypsin inhibitor, this could mean CC was involved in a protective response, possibly by reducing ECM degradation. This theory was supported by the significant levels of CC in areas of disorganized lung architecture. Similarly, some mammalian CRISP proteins are found loosely associated with sperm and have been indicated in sperm–oocyte fusion (Evans 2002). As oocytes are surrounded by a matrix containing hyaluronan (a proteoglycan) (Richards 2005), there is potential that these interactions could be linked to CRISP-related ECM remodelling.

An important feature of this secreted protein is its regular appearance in the blood vessels and in the lung lining. If CC can be detected in blood or bronchoalveolar lavage (BAL), it has good potential as a biological marker for the detection of oedema and inflammation (early fibrotic changes) in the lung. The ability to screen blood or lavage samples (rather than tissue samples, requiring a biopsy for gene analysis) increases the possibility of creating screening or diagnostics tools for clinical applications. At the very least, it might prove useful for detecting bleomycin-induced injury in chemotherapy patients.

In conclusion, CC has many potential mechanisms involving resistance to the development of pulmonary fibrosis, and the defence of the lung to pathogens. The appearance of elevated levels of CC in the blood and possibly BAL, or even sputum or breath condensate, gives it great potential as a clinical marker for early bleomycin-induced lung injury.

Acknowledgements

The authors would like to thank Dr Ilyas Kahn for donating the Cocoacrisp antibody which made this study possible. Thanks also to AstraZeneca for funding this research.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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