Soluble Endostatin is a novel inhibitor of epithelial repair in Idiopathic Pulmonary Fibrosis

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Abstract 247

Aberrant angiogenesis and defective epithelial repair are key features of Idiopathic pulmonary fibrosis (IPF). Endostatin is an anti-angiogenic peptide with known effects on endothelial cells. This study aimed to establish the levels of endostatin in the bronchoalveolar lavage fluid (BALF) in IPF and to investigate its actions on distal lung epithelial cells (DLEC) and primary type II cells.

Methods: 20 patients with IPF and 10 controls underwent BAL. Endostatin was measured by ELISA. BALF cytokines and Matrix Metalloproteinase (MMP) -3 were measured by Luminex array. Primary DLEC monolayers were wounded and treated with endostatin. Apoptosis and cell viability were assessed.

Results: Endostatin was elevated in the BALF and plasma of patients with IPF compared with normal controls. There was a negative correlation between endostatin, forced vital capacity and gas transfer. Endostatin correlated with a number of pro-inflammatory cytokines and MMP-3. Physiological endostatin doses inhibited DLEC wound repair by 44% in an effect that was partially FasL and caspase dependent. Endostatin increased apoptosis rates by 8% and reduced their viability by 34%. Similar effects of endostatin were seen in primary type II cells in terms of inhibition of wound repair and proliferation.

Conclusions: Elevated BALF endostatin levels correlated with a number of elevated cytokines, MMP-3 and lung function in IPF. Endostatin is a novel inhibitor of DLEC wound repair, inducing apoptosis and reducing cell viability in a FasL and caspase dependent manner. Endostatin may play a role in aberrant epithelial repair in IPF.
Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease of unknown aetiology (1). Current theories speculate that IPF results from abnormal wound healing in response to multiple, microscopic sites of alveolar epithelial cell (AEC) injury, activation and apoptosis (2). AEC apoptosis has been implicated in the initiation of fibrotic foci (3;4) and an increased rate of apoptosis could contribute to the loss of balance in cell turnover and abnormal re-epithelialization. Caspase inhibition blocks AEC apoptosis and reduces the accumulation of collagen matrix in animal models (5).

Collagens are a family of extra-cellular matrix (ECM) proteins that play a dominant role in maintaining the tissue structural integrity. Excessive collagen deposition leads to fibrosis disrupting the normal functioning of surrounding tissues (6). Collagen XVIII is a major proteoglycan found in alveolar capillary and epithelial basement membranes. Proteolytic enzymes cleave peptide bonds within the protease sensitive hinge region of collagen XVIII to release anti-angiogenic endostatin fragments varying in size between 20 and 30 kDa (7). Endostatin has been implicated in the microvascular damage found in pre-eclampsia and can be used to suppress tumour neovascularization/angiogenesis in mice (8). Endostatin levels have previously been found to be elevated in the serum of patients with IPF (9).

Endostatin inhibits proliferation and migration of endothelial cells by interruption of focal adhesions and actin stress fibres, essential to cell motility (10). Endostatin induces endothelial cell apoptosis (11) and has been shown to associate with \( \alpha_5\beta_1 \) integrin,
essential for endothelial cell proliferation and apoptosis (12), and VEGFR-2(13). Given the importance of the epithelium in IPF and the fact AEC express both α5β1 integrin(14) and VEGFR-2(15), we hypothesized that endostatin may have a role in inhibiting AEC repair in patients with IPF.

Matrix metalloproteinases (MMP) are essential for ECM remodeling, wound repair, and angiogenesis and have been implicated in the pathogenesis of IPF. MMP-3 (stromelysin-1) has a wide range of actions influencing fibrinolysis, angiogenesis (16) and is able to cleave endostatin from Collagen XVIII(17). Establishing MMP-3 levels would give an indication of the potential for local proteolytic cleavage of endostatin.

The relationship between endostatin and cytokine networks is undetermined, but it is known endostatin is able to alter expression of a wide range of genes(18). CXC cytokines that contain the terminal glu-leu-arg (ELR+ve) motif are potent induces of angiogenesis. Cytokines that don’t present this motif (ELR-ve) which are mainly IFN inducible are in contrast anti-angiogenic. An imbalance in pro and anti-angiogenic CXC cytokines, has been described in IPF(19).

The study aims were to determine bronchoalveolar lavage fluid (BALF) and plasma endostatin levels in IPF and WG and then relate endostatin to lung function and alveolar cytokine levels. In vitro experiments were designed to establish whether physiological levels of endostatin can influence human primary distal lung epithelial cell actions and propose a potential mechanism of action.
Methods

Subjects

20 patients with IPF diagnosed according to current American Thoracic Society (ATS) criteria were recruited from the specialist Interstitial Lung Disease clinic at University Hospital Birmingham, UK. Open lung biopsy confirmed Usual Interstitial Pneumonia in 5 patients where diagnosis was uncertain. Bronchoscopy was performed during the investigation stage after referral, before the start of definitive treatment. 10 healthy individuals free from respiratory disease were recruited as controls. This study was approved by the local ethical committee and patients gave written informed consent.

Measurements

Patients underwent bronchoscopy and bronchoalveolar lavage (BAL) as described previously(20). Endostatin was measured in BALF and plasma by ELISA kit (R&D) according to manufacturer’s instruction. BALF cytokines (IL-8, ENA78, IL1RA, IL-6, MCP-1, IFNγ) and MMP-3 were measured by Luminex array (R&D systems).

Protein was measured using the Bio-Rad DC protein assay kit II. The protein permeability index (PPI) was calculated as the ratio of BALF to plasma protein as described previously(21).

Pulmonary function testing

Forced vital capacity (FVC) was measured using the Jaeger Compact system (Viasys Healthcare). Total lung diffusing capacity for carbon monoxide (TLCO) was measured
by single-breath technique (Jaeger Compact system). Results were expressed as the percent of predicted values.

**Cell culture**

Primary human distal lung epithelial cells (DLEC) (Cambrex) were cultured in complete growth media (SAGM, Cambrex), according to manufacturer’s protocol. Cells were obtained from 3 separate donors and experiments were performed before passage 3. Confluent DLEC monolayers were wounded with a 2mm mechanical wound as described previously(22). The wounds were photographed under microscope at 0 and 18 hours and analysed with Scion image software.

A dose response of 0-1000ng/ml of endostatin (Molecular Probes) on DLEC wound repair was assessed. Experiments were then repeated by incubating endostatin alone or pre-incubating with anti-FasL antibody (1ng/ml)(R&D) or the caspase inhibitor Z-DEVD-FMK (20µM)(R&D).

**Actions of endostatin on DLEC apoptosis and cell viability**

DLEC apoptosis was assessed using annexin/ propidium iodide fluorochromes (Invitrogen) and analysed by flow cytometer (Coulter EPIC flowcytometer) and Cellquest software. This experiment was repeated with anti-FasL antibody or after pre-incubating the monolayer with the caspase inhibitor Z-DEVD-FMK as above. Cellular viability was measured using Cell titre (Promega) according to manufacturer’s instructions.
Alveolar type II cell extraction and culture

In order to assess the effects of endostatin upon ATII cells lung samples were obtained from 4 patients undergoing lung resection for lung cancer, ATII cells were extracted according to the method of Witherden and Tetley(23). Average yields of ATII were 30 million cells per resection with a purity of 92%. Cells were tested for ATII cell phenotype by alkaline phosphatase staining, lysotracker lamellar body staining and by PCR expression of Surfactant protein C – a type II cell marker with negative expression of Aquaporin V (a type I cell marker) (data not shown). For viability experiments, cells were used 24 hours after extraction. For wound repair experiments, 0.5 million cells were seeded onto 24 well plates and grown for 4 days in DCCM (Troon Scientific,Troon) media supplemented with 10% fetal calf serum. Wound repair assays were performed as per DLEC methods above. BRDU incorporation of ATII cells was assessed using a colorimetric assay (Calbiochem, UK) according to manufacturers’ instructions. To allow for variability of basal proliferation between batches of ATII cells, cells were stimulated with 10ng/ml TNF-alpha for 1 hour prior to addition of BRDU and results expressed as % control BRDU incorporation.

Statistics

Normally distributed data, assessed by Kolmogorov-Smirnoff test is presented as mean ± standard error (SE). Comparison between two groups was analyzed using Student’s t-test and multiple groups by one-way ANOVA with Tukey’s post hoc analysis. Non-parametric data is presented as median and interquartile range (IQR). Between group comparisons were performed using the Mann Whitney U test; multiple groups using the
The cytokine tests had a Bonferroni correction applied; a p value <0.005 as opposed to p ≤ 0.05 for all other data, was considered significant. Correlations were made using Spearman rank. This study was considered hypothesis generating so a power calculation was not performed. Statistics were performed using SPSS 15.

**Results**

**Demographics**

20 patients with IPF and 10 healthy controls were recruited. A summary of patient characteristics is shown in table 1. 6 patients agreed to a sequential BAL 6 months after starting treatment with Prednisolone and azathioprine +/- N-acetylcysteine. The mean age of IPF patients was higher than healthy controls however there was no relationship between BALF endostatin and age (rho=-0.161, p=0.443).

<table>
<thead>
<tr>
<th>Table 1: Demographics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient numbers</strong></td>
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<tr>
<td>20</td>
</tr>
<tr>
<td><strong>Age (range)</strong></td>
</tr>
<tr>
<td><strong>Sex: Male</strong></td>
</tr>
<tr>
<td><strong>Smoking status: current, ex, never</strong></td>
</tr>
<tr>
<td><strong>Pack years (IQR)</strong></td>
</tr>
<tr>
<td><strong>BAL total cell count x10⁴/ml (IQR)</strong></td>
</tr>
<tr>
<td><strong>BAL neutrophil percentage (IQR)</strong></td>
</tr>
<tr>
<td><strong>FVC (IQR)</strong></td>
</tr>
</tbody>
</table>
FVC - forced vital capacity, TLco – Carbon monoxide diffusion factor (both expressed as percentage of normal). Data expressed as median (IQR).

**BALF and Plasma levels of Endostatin are elevated in patients with IPF**

Endostatin was detectable in the BALF and plasma of all patients and normal control samples. Endostatin was significantly elevated in both BALF (IPF [mean 1.32 ng/ml, SE 0.20], normal [0.12 ng/ml, SE 0.03], p<0.001) and plasma (IPF [mean 259.2 ng/ml, SE 41.3], normal 94.7 ng/ml, SE 18.9] p<0.001) of IPF patients compared to controls (figure 1a). Plasma endostatin levels correlated with BALF endostatin (r=0.336, p=0.007). Endostatin levels remained elevated in the six patients who underwent sequential BAL after treatment; first BALF average endostatin 1.02ng/ml SE 0.29, second BALF 1.42ng/ml, SE 0.29 (p=0.792) (data not shown).

**BALF endostatin correlates with markers of lung function**

BALF endostatin negatively correlates with FVC (r=-0.604, p=0.006) and TLco (r=-0.612, p=0.005). BALF neutrophil % also correlated with FVC (r=-0.627, p=0.004) and Kco (r=-0.528, p=0.02). BALF endostatin correlates with BALF protein (r=0.740 p=0.001) and the protein permeability index (r=0.396, p=0.05).
**BALF cytokine levels**

A number of BALF cytokines are elevated compared with normal controls, including the ELR+ve cytokines ENA-78 and IL-8 (Table 2).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal controls</th>
<th>IPF</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
</tr>
<tr>
<td>ENA-78</td>
<td>0.01</td>
<td>16.53</td>
<td>328.6</td>
</tr>
<tr>
<td>IL-8</td>
<td>25.9</td>
<td>54.4</td>
<td>338.2</td>
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<td>IFNγ</td>
<td>0.2</td>
<td>6.93</td>
<td>6.3</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>945.7</td>
<td>1696.7</td>
<td>7358.4</td>
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<tr>
<td>MCP1</td>
<td>15.7</td>
<td>7.46</td>
<td>341.8</td>
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<tr>
<td>MIP1α</td>
<td>65.5</td>
<td>129.5</td>
<td>145.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.01</td>
<td>12.27</td>
<td>16.4</td>
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<tr>
<td>IL-6</td>
<td>0.37</td>
<td>0.5</td>
<td>7.7</td>
</tr>
<tr>
<td>RANTES</td>
<td>6.1</td>
<td>36.5</td>
<td>39.8</td>
</tr>
<tr>
<td>G-CSF</td>
<td>22.2</td>
<td>13.7</td>
<td>55.1</td>
</tr>
</tbody>
</table>

**Table 2: BALF cytokine levels:**

Cytokines were measured using a luminex™ array. Results were compared by Mann Whitney U test between IPF patients and healthy controls. After Bonferroni correction a p value <0.005 is considered significant.
Correlations between BALF endostatin and cytokines

BALF endostatin correlated with the ELR+ve cytokines IL-8 (rho 0.562, p<0.001) and ENA-78 (rho=0.501, p=0.008), neutrophil % (rho=0.508, p=0.001) and the cytokines IL-1ra (rho=0.726, p<0.001), MCP-1 (rho=0.512, p=0.005) and IL-6 (rho=0.607, p=0.008).

BALF levels of MMP-3 are elevated in IPF and correlate with BALF endostatin.

Median IPF BALF MMP-3 levels were 24.8 pg/ml (IQR 32.2) compared to 9.71 pg/ml (IQR 5.2) in normal individuals (p=0.009) (figure 1b). BALF endostatin correlated with BALF MMP-3 supporting a role for MMP-3 in endostatin release from type XVIII collagen in IPF (rho=0.45, p=0.033) (data not shown). MMP3 levels also correlated with neutrophil percentage (r=0.424, p=0.039), IL-8 (rho=0.625, p=0.001) and G-CSF (rho=0.599, p=0.003). There was no relationship with ENA-78 or IFNγ.

Endostatin effects on DLEC wound repair, apoptosis and cell viability

Endostatin inhibited wound repair in DLECs (figure 2a) and this effect was partially blocked by FasL antibody and the caspase inhibitor Z-DEVD-FMK; control (56.58% [SE 2.1]), endostatin 100ng (42.52% [SE 0.65]), endostatin + anti-FasL antibody (54.21% [SE 4.44]), endostatin + Z-DEVD-FMK (49.2% [SE 0.79]), Anova p<0.001). There was a significant difference between control and endostatin (p<0.0001), between endostatin and endostatin + anti-FasL antibody (p=0.029) and endostatin + Z-DEVD-FMK (p=0.001) (figure 2b). At higher endostatin doses endostatin often increased the measured wound which is described as a negative percentage wound repaired.
Apoptosis rates were higher in endostatin treated cells than controls (100ng 58.8% [SE 0.98], 1000ng 58.9% [SE 0.81], controls 50.8% [SE 1.21], Anova p<0.001 and Tukey’s p<0.001, p<0.001 respectively) (figure 3a). The affect of endostatin on DLEC apoptosis was attenuated by anti- FasL antibody (mean 56.9% [1.0] v 45.7% [4.4] p=0.024). Caspase inhibition reduced the endostatin effect but this did not achieve statistical significance (mean 56.9% [1.0] v 50.4% [1.3], p=0.289) (data not shown).

Cell titre colorimetric assay showed reduced cell viability in endostatin treated DLEC cells and that this effect was enhanced at a higher dose (p<0.001) (figure 3b).

**Effect of endostatin upon Primary human ATII cell wound repair and proliferation.**

Endostatin caused a dose dependent reduction in wound repair after 48 hours (fig 4a). Unlike in DLEC, endostatin did not reduce cellular viability over 24 hours suggesting that in ATII this action was not associated with cell death (data not shown). To confirm that the effects of endostatin on wound repair were related to proliferation we assessed ATII cell BRDU incorporation. Endostatin inhibited BRDU incorporation in a dose dependent manner over 36 hours (control (100% IQR 100-101.42), endostatin 10 ng/ml (82.5% (IQR 61.75-84.5, p=0.034), endostatin 100 ng/ml (71.5% (IQR 52.5-86), p=0.03), and endostatin 1000ng/ml (57% control incorporation (IQR 42.25-76.25), p=0.029) (fig 4b)
Discussion

This is the first study to demonstrate endostatin in the alveolar space of patients with IPF and that levels are elevated compared with normal controls. The relationship between BALF endostatin and physiological severity indicates a potentially important clinical role for endostatin in IPF. In addition to its known inhibitory effects on endothelial cells, this study confirms actions of endostatin on DLEC and ATII wound repair. These effects on DLEC appear to involve apoptosis, be partially FasL dependent and can be abrogated by caspase inhibition.

Two forms of collagen XVIII are expressed in normal human tissues, designated the SHORT and LONG variants(24). The LONG form appears to be largely produced by hepatocytes in the liver sinusoids and is a measurable as a plasma protein. The SHORT form is expressed in most vascular and epithelial basement membranes, including the specialized capillaries found in lung alveoli. The carboxy terminus of type XVIII collagen includes a hinge region that displays protease-sensitive sites which generate the endostatin fragments. A number of enzymes, including neutrophil elastase, cathepsins, and matrix metalloproteinases (including the fibroblast derived MMP-3) have been implicated in the pathogenesis of IPF(6). These proteases are known to act upon this hinge region to release not only endostatin but other larger fragments that contain the endostatin fragment. Previous studies have suggested that these C-terminal fragments may have similar anti-angiogenic actions to endostatin(17).
Endostatin levels in the IPF lung may be elevated because of increased local cleavage of alveolar collagen XVIII. Gene expression array data suggests an increase in type XVIII collagen mRNA in whole lung extracts from IPF patients (25) and in an animal model of FasL induced lung injury there is upregulation of type XVIII collagen mRNA (26). Although our correlations between MMP-3 and endostatin do not prove a mechanistic link, taken together with our findings of increased BALF MMP-3 in IPF, this data suggests that local intra-alveolar degradation of type XVIII collagen may be important in endostatin generation. The proteolytic cleavage of collagen XVIII may only partially govern endostatin levels as has been shown that endothelial endostatin release may be induced by general cell stress and can be modulated by the nitric oxide/cGMP pathway (27). A wide range of inflammatory cytokines were measured in this study and found to correlate with endostatin suggesting endostatin generation may be up regulated by an alveolar inflammatory milieu. Alternatively the observed relationship between BALF endostatin and protein permeability, and the plasma: BALF endostatin gradient suggests that alveolar levels of endostatin may be raised due to leakage from the microvasculature into the alveolus at sites of increased alveolar barrier permeability. Given the anti-endothelial cell actions of endostatin, endostatin may play a pathophysiological role in this abnormal permeability.

Prior to this study the main focus of research into the biological effects of endostatin was upon endothelial cells in angiogenesis and cancer biology. The actions of endostatin on endothelial cells has variously been shown to reduce migration, spreading and induce apoptosis (28;29). Not all studies have confirmed these effects and often these experiments have used supra-physiological doses. This study described the actions of
endostatin on epithelial cells for the first time. Physiological doses of endostatin significantly reduced both DLEC and ATII cell wound repair with inhibitory effects on cellular viability and increased apoptosis in DLEC. These actions upon DLEC were partially mediated by FasL and caspase pathways, this is relevant as FasL induced epithelial apoptosis has been implicated in the pathogenesis of IPF. Endostatin is known to promote the formation of lipid rafts essential for signalling through the Fas death receptor(30) and provides a potential mechanism whereby endostatin induces epithelial apoptosis in our patients. The relatively small increase in DLEC apoptosis (figure 3a) caused by endostatin, suggests additional mechanisms maybe involved in endostatin inhibition of DLEC wound repair. Spreading and migratory processes are essential for monolayer repair and the effects of endostatin on these mechanisms warrant further investigation.

It has been suggested that disordered epithelial remodeling promotes fibroproliferation in IPF. Endostatin can interact with both integrins and VEGFR-2 which are known to be present upon epithelial cells (12). The known effects of endostatin upon WNT and cyclin D1 expression may be important in its epithelial effects since pulmonary epithelial cell turnover is also regulated by these signaling pathways(31). Further work to characterize the mechanism of endostatin action upon epithelial cells is required. Nevertheless our study suggests that there may be a direct link between collagen degradation and ongoing epithelial cell apoptosis induced by endostatin production in patients with IPF.

This study has several limitations. Firstly our patient population did not all have lung biopsies to prove UIP although they were well characterised according to international
guidelines in a specialist clinic. Furthermore, the difference in age between the IPF patients and our healthy controls may be a confounding factor when comparing endostatin levels. To address this we examined the relationship between age and endostatin levels in IPF and normal controls and found no correlation. Finally we are unable to assess the relative importance of endostatin within BALF in an angiogenesis bioassay due to the current lack of an effective inhibitor of endostatin bioactivity.

In conclusion, this study has demonstrated elevated endostatin levels in IPF patients. Endostatin levels correlated with the degree of lung function impairment and levels of inflammatory mediators that are associated with angiogenesis. Endostatin has been found to inhibit primary epithelial cell wound repair by mechanisms that involve both increased apoptosis rates or inhibition of proliferation. In conjunction with its anti-angiogenic properties therefore, the presence of endostatin within the lung in IPF may result in defective alveolar epithelial repair. This study raises the possibility of an endostatin inhibitor as a therapeutic option in IPF.

**Figure Legends**

**Figure 1: Endostatin levels in IPF and controls**
Graph A: Endostatin levels in the BALF of IPF (n=20) and healthy controls (n=10)  
Graph B: MMP-3 levels in the BALF of IPF (n=20) and healthy controls (n=10).

**Figure 2: Endostatin inhibits DLEC wound repair**
Graph A: Endostatin inhibited DLEC wound repair in a dose responsive manner (n=6 at each dose).
Graph B: This graph shows that the effects of endostatin (100ng) can be partially inhibited by anti FasL and the Caspase 3 inhibitor Z-DEVD-FMK (Anova p=0.007) (n=6 for each experiment).

Figure 3: Endostatin affects on DLEC apoptosis and cell viability
Graph A: Endostatin increases apoptosis of DLEC cells at 100 and 1000ng doses (n=6 at each dose).
Graph B: Endostatin reduces cell viability in a dose responsive manner (n=16 at each dose).

Figure 4: Endostatin impairs human ATII wound repair and proliferation
Graph A: Endostatin inhibits human ATII wound repair in a dose dependent manner, anova p=0.001 (8 replicates at each dose from each of 4 lung tissue resections).
Graph B: Endostatin inhibited ATII cell proliferation as measured by BRDU incorporation (6 replicates at each dose from each of 4 lung tissue resections).

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Reference List


Figure 1

Graph A

Graph B

p = 0.001

p = 0.009
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