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VCAM-1 is a TGF-β1 inducible gene upregulated in idiopathic pulmonary fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a chronic lethal interstitial lung disease of unknown etiology. We previously reported that high plasma levels of vascular cell adhesion molecule 1 (VCAM-1) predict mortality in IPF subjects. Here we investigated the cellular origin and potential role of VCAM-1 in regulating primary lung fibroblast behavior. VCAM-1 mRNA was significantly increased in lungs of subjects with IPF compared to lungs from control subjects ($p = 0.001$), and it negatively correlated with two markers of lung function, forced vital capacity (FVC) and pulmonary diffusion capacity for carbon monoxide (DL_{CO}). VCAM-1 protein levels were highly expressed in IPF subjects where it was detected in fibrotic foci and blood vessels of IPF lung. Treatment of human lung fibroblasts with TGF-β1 significantly increased steady-state VCAM1 mRNA and protein levels without affecting VCAM1 mRNA stability. Further, cellular depletion of *VCAM-1* inhibited fibroblast cell proliferation and reduced G2/M and S phases of the cell cycle suggestive of cell cycle arrest. These effects on cell cycle progression triggered by *VCAM1* depletion were associated with reductions in levels of phosphorylated extracellular regulated kinase 1/2 and cyclin D1. Thus, these observations suggest that VCAM-1 is a TGF-β1 responsive mediator that partakes in fibroblast proliferation in subjects with IPF.

1. Introduction

Idiopathic pulmonary fibrosis is a chronic lethal lung disease with unknown cause and no cure [1,2]. IPF has the worst prognosis among interstitial lung diseases (ILD) [3] with a median survival of 2.5 to 3 years [4]. The principal hypothesis of IPF pathogenesis has been suggested to be aberrant alveolar-re-epithelialization caused by repeated alveolar epithelial injury [5]. The failure of proper wound healing is proposed to be due to a complex array of mechanisms involving modifications of fibroblast–myofibroblast transformation, epithelial cell apoptosis, changes in hemostasis of cytokines, chemokines, and transforming growth factor β1 (TGF-β1), all of which play critical roles in IPF pathogenesis via promoting extracellular matrix accumulation and phenotypic changes of fibroblasts and epithelial cells in IPF lungs [6–8].

Vascular cell adhesion molecule 1 (VCAM-1) is an immunoglobulin superfamily member [9] that is expressed in large and small blood vessels after cytokine stimulation and mediates adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. VCAM-1 plays a critical role in the development of atherosclerosis and rheumatoid arthritis [10], facilitates leukocyte-endothelial cell adhesion and plays a role in signal transduction [11]. We previously reported that high VCAM-1 protein in peripheral blood levels of IPF patients predicts mortality [12], but its mode of expression and putative role as a contributor to IPF pathogenesis remains unclear. In some studies there has been limited success in detecting levels of VCAM-1 protein in IPF lungs [13].

In this study, we demonstrate that VCAM-1 expression is elevated in human IPF lung fibroblasts and that its expression is responsive to actions of a critical mediator of disease, TGF-β1. Interestingly, silencing *VCAM-1* mRNA inhibits fibroblast proliferation and impairs cell cycle progression through depletion of specific signaling factors implicating in cellular proliferation. In aggregate, these observations provide a foundation for further studies on the mechanistic role of VCAM-1 in IPF pathogenesis.

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2. Materials and methods

2.1. Materials

VCAM-1 antibody was obtained from Novus Biologicals (Littleton, CO). Anti-collagen type 1 antibody was from Rockland (Limerick, PA). β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). The cyclin D1, cyclin D2, cyclin D3, cdk2, cdk4 and cdk6 antibodies were from Cell Signaling (Danvers, MA). The anti-ERK1/2, phospho-ERK1/2, p38 and phospho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The miRNA mini Kit was from Qiagen (Louisville, KY). The primers for VCAM-1 and qRT-PCR were purchased from ABI (Foster City, CA). Recombinant human TGF-β was obtained from R&D Systems (Minneapolis, MN). VCAM-1 shRNA was purchased from Dharmacon (Lafayette, CO). The CytoSelect BrdU cell proliferation ELISA kit was from Cell Biolabs (San Diego, CA). The Cell Cycle Phase Determination Kit was obtained from Cayman (Ann Arbor, MI). Western Lightning Plus ECL was from PerkinElmer (Boston, MA). Actinomycin D was from Sigma (St. Louis, MO).

2.2. Microarrays

Primary lung tissues were isolated from normal ($n = 109$) and IPF lungs ($n = 134$). Total RNA was extracted, labeled and hybridized to Agilent 44 k whole human genome microarrays (Agilent Technologies, Wilmington, DE). After cyclic-LOESS normalization, Genomica and SAM were applied for statistical analysis. A 5% false discovery rate was used for significance in microarray data.

2.3. RNA extraction and real-time PCR analysis

All patients were evaluated at the University of Pittsburgh Medical Center, Pittsburgh, PA; the study was performed in accordance to the protocols approved by the University of Pittsburgh Institutional Review Board. The diagnosis of IPF was established on the basis of American Thoracic Society (ATS) and European Respiratory Society (ERS) criteria [14]. Clinical data were available through the Simmons Center Database at the University of Pittsburgh. Smoking status was defined as previously described [15]. All patients signed informed consent to participate in the study. Total cellular RNA was extracted from the lungs of eleven IPF and eleven control subjects obtained from University of Pittsburgh Health Sciences Tissue Bank (Pittsburgh, PA). RT-PCR was performed according to the manufacturer's protocol as we described previously [16].

2.4. Immunohistochemical analysis

Paraffin-embedded IPF and control lung tissue specimens were obtained from the University of Pittsburgh Health Sciences Tissue Bank (Pittsburgh, PA), and immunohistochemical analysis was performed as previously described [17]. Briefly, the slides were deparaffinized in xylene, ethanol, and rehydrated in phosphate-buffered saline (PBS), blocked for 1 h in 10% bovine serum albumin and incubated with primary anti-VCAM-1 antibody overnight. Slides were then washed with PBS three times and incubated with biotinylated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h. The images were visualized with an Olympus CH2™ microscope and obtained using aDP25 camera (Olympus America Inc., Melville, NY).

2.5. Cell culture, TGF-β1 stimulation and immunoblot analysis

Early passages (1–3) of primary normal human lung fibroblasts from the University of Pittsburgh Tissue bank were cultured in F-12 (1×) medium supplemented with 10% fetal bovine serum (FBS) according to the supplier's protocol. All experiments were performed on cells at 70–80% confluence. Treatment with recombinant TGF-β1 (5 ng/ml) was performed for 24 h. Immunoblots were performed with antibodies against VCAM-1 (1:1000), β-actin (1:10,000) and bands quantitated and analyzed using ImageJ software.

2.6. Enzyme-linked immunosorbent assay (ELISA)

IPF subjects ($n = 48$) and control subjects ($n = 50$) were evaluated at the University of Pittsburgh Medical Center. Recruitment and data collection has been previously described [18]. Smoking status was defined as described in [15]. The demographic and clinical information of subjects involved in this study are shown in Table 1 from the Lung Tissues Research Consortium (LTRC) for the gene expression cohort and Table 2 for the Pittsburgh plasma cohort. VCAM-1 levels were detected using a VCAM-1 ELISA kit according to the manufacturer's protocol.

2.7. Cell proliferation

The proliferation of lung fibroblasts was detected using a CytoSelect BrdU cell proliferation ELISA kit. To determine BrdU incorporation into cellular DNA during cell proliferation, cells on a 96-well cell culture plate were transfected with *VCAM-1* shRNA or a control shRNA using Effecten transfection reagent. After 12 h, medium was changed and cells were incubated for an additional 24 h. An aliquot of BrdU was then added to the medium and cells were incubated for an additional 3 h at 37 °C. After washing with PBS, cells were fixed for 30 min and BrdU incorporation into total cellular DNA was determined using anti-BrdU antibody following the manufacturer's instructions.

2.8. Cell cycle phase determination

To determine cell cycle progression, the lung fibroblasts were transfected with *VCAM-1* shRNA or control shRNA as described above [19,20]. After transfection, cells were fixed and then stained with propidium iodide for 30 min at room temperature in the dark. Cells in individual cycle phases were analyzed by flow cytometry and captured with a 488 nm excitation laser.

2.9. Bleomycin murine model of fibrosis

Male and female C57BL/6 mice (6 to 8 weeks old) are deeply anesthetized and bleomycin at 3 U/kg (standard dose) or 1 U/kg (low dose) or saline control was administered intratracheally in a volume of 50 μL. Mice are sacrificed on day 14 with pentobarbital, and the lungs are excised for determination of VCAM-1 content. All procedures were executed in accordance with approved protocols through the University of Pittsburgh Institutional Animal Care and Use Committee.

Table 1
The demographic and clinical information of subjects from the Lung Tissues Research Consortium.

LTRC lung cohort	Control	IPF	*P*-value
Age	63.6 ± 11.4	63.9 ± 8.2	NS
Female	27 (42.3%)	21 (25%)	NA
Non-Caucasian	8 (7.33%)	9 (6.7%)	NA
FEV1%	95.0 ± 12.62	70.4 ± 18.2.6	1.03E−25
FVC%	94.4 ± 13.12	63.38 ± 16.16	3.55E−38
DLCO %	84.05 ± 16.7	46.7 ± 17.8	5.96E−38

1)FVC = force vital capacity.
2)DLco = carbon monoxide diffusing capacity.
3)And FEV1 = forced expiratory volume in one second.

Table 2
The demographic and clinical information of subjects from the Pittsburgh plasma cohort.

Pittsburgh plasma cohort	Characteristics	IPF
Age	Years	64.8 ± 8.2
Female Sex	Absolute/PEZRCENT %	17 (35.4%)
Non-Caucasian	Absolute number	3(6.25%)
PFTs	FEV1%	77.2 ± 18.8%
	FVC%	68 ± 19.8%
	DLCO%	48.2 ± 11%

4)FVC = force vital capacity.
5)DLco = carbon monoxide diffusing capacity.
6)FEV1 = forced expiratory volume in one second.

2.10. Statistical analysis

The group comparisons between diseased and control subjects were performed using an unpaired two-tailed Student's *t*-test for normally distributed data. A level of $p < 0.05$ was considered statistically significant. Spearman correlation tests were used for computation of VCAM-1 mRNA correlation with pulmonary function tests utilizing Stata software. The bands on immunoblots were quantified by using ImageJ, and then densitometric ratios were calculated. The ratios data were statistically analyzed to discriminate their differences by using an unpaired student *t* test. The immunoblot data were representatives of 3–5 separate experiments.

3. Results

3.1. VCAM-1 mRNA levels in IPF subjects negatively correlate with pulmonary function

Data mining of our previously published LTRC microarray data revealed that VCAM1 is one of the most significantly expressed genes in IPF lung [21,22]. Indeed, the steady-state VCAM-1 mRNA levels were significantly increased in IPF lungs compared to controls ($P = 0.0001$) (Fig. 1A). Analysis of potential correlation of VCAM-1 mRNA levels with pulmonary function parameters revealed that there is a statistically significant negative correlation between VCAM-1 and DLco percent predicted (rho = −2, $P = 0.02$) and FVC percent predicted (rho = − 0.22, $P = 0.0121$) (Fig. 1B and C, respectively) demonstrating that increased VCAM-1 mRNA negatively correlates with pulmonary function. To validate the microarray results, we performed VCAM-1-specific qRT-PCR analysis of the independent cohort consisting of 11 IPF and 11 control lung specimens. In agreement with microarray data, VCAM-1 mRNA was significantly increased in IPF lungs compared to controls (Fig. 1D). Taken together, these data demonstrate that VCAM-1 mRNA is increased in IPF lung that correlates with worse pulmonary function.

3.2. VCAM-1 protein levels are elevated in IPF lungs

To determine whether increased *VCAM-1* mRNA levels result in elevated expression of protein, we next compared VCAM-1 protein levels in the plasma and lungs of IPF and non-diseased (control) subjects. Comparison of plasma from 48 IPF and 50 control subjects using VCAM-1 ELISA assays demonstrated that VCAM-1 protein levels were significantly higher in the IPF cohort (Fig. 2A). Levels of immunoreactive VCAM-1 were almost undetectable at baseline in control lungs. Similarly, analysis of whole lung tissue from four IPF and four control subjects showed a 3-fold increase in VCAM-1 protein levels in IPF lungs (Figs. 2B, C). To confirm our findings, we performed analysis of whole lung tissues from mice with bleomycin-induced lung fibrosis. We found that VCAM-1 levels were modestly increased in bleomycin-treated mice compared to control lungs (Fig. 2D). Collectively, these data demonstrate that the VCAM-1 protein levels are elevated in both the plasma and lungs of IPF subjects and in a murine lung model of fibrosis.

3.3. VCAM-1 expression, cellular localization and regulation by TGF-β1

To determine the cell types responsible for VCAM-1 expression in IPF lungs, we performed immunohistochemical analysis of lung tissue

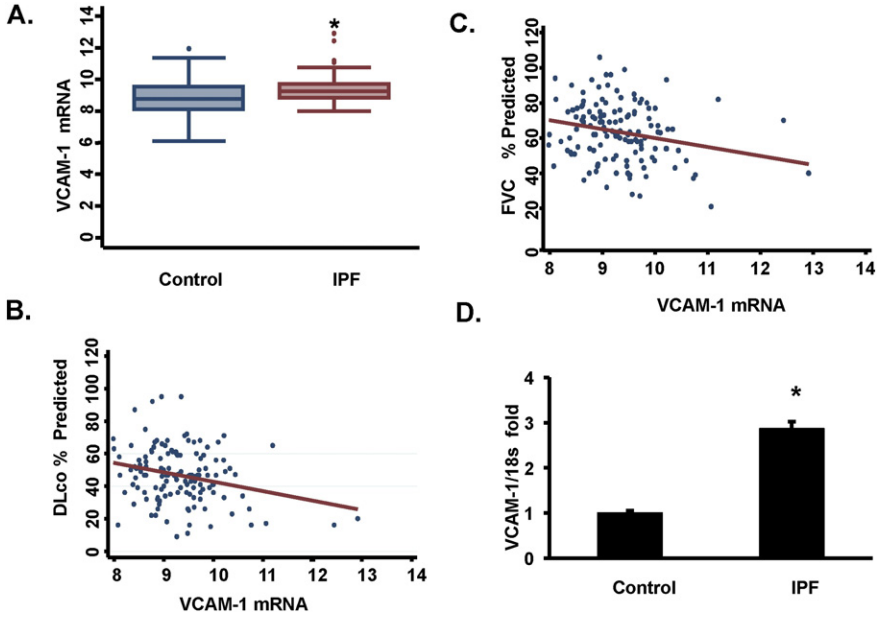

Fig. 1. VCAM-1 mRNA levels are increased in IPF lungs and negatively correlate with pulmonary function. A. VCAM-1 expression levels were extracted from publically available data by microarray profiling of lung tissues from IPF (IPF: $n = 134$) and control subjects (control: $n = 109$). VCAM-1 is up-regulated in IPF subjects compared to control (*$P = 0.0001$) [21,22]. B, C: Associations between continuous variables were established by a Spearman correlation analysis and the regression lines have been fitted in two-way scatterplots by Stata software. For FVC association; rho = −0.2186, $P = 0.0123$ and for DLco; rho = −0.2074, $P = 0.02$. D: *VCAM-1* mRNA levels in lungs of control subjects ($n = 11$) and IPF subjects ($n = 11$) were measured using qRT-PCR analysis. Total cellular RNA was extracted with Triazol. The mitochondrial ribosomal protein S18A (MRPS18A) was used as an internal standard. Data represent fold change in *VCAM-1*/*S18A* ratio with a ratio for control cells taken as one fold. Data are expressed as means ± SE using an unpaired students *t*-test *$P < 0.003$ for controls vs IPF groups.

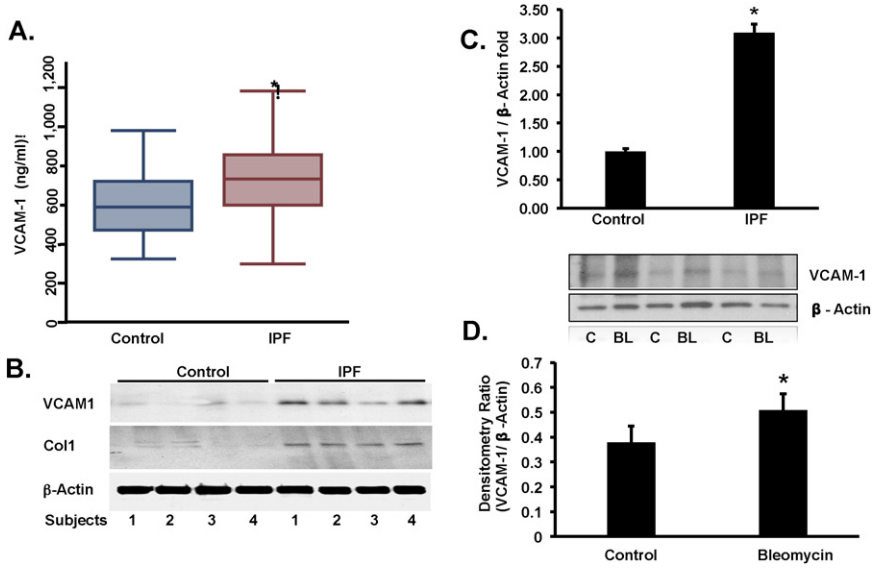

Fig. 2. Plasma VCAM-1 levels are increased in IPF subjects. A. Plasma VCAM-1 (ng/ml) was assayed in control subjects ($n = 50$) and IPF ($n = 48$) subjects using an ELISA assay. Approximately equal sample sizes were used in each group and the comparison between each group was computed using an unpaired Student t-test. ($P = 0.0003$, mean = 737.2979 and SD = 186.8587 for IPF and mean = 596.735 and SD = 178.8378 for controls). B, C. Whole lung tissue lysates were obtained from IPF lungs and control lungs as described in the Materials and methods section. Protein levels of VCAM-1 and collagen 1 (Col1, positive control) were determined by immunoblotting. Shown is a representative immunoblot for VCAM1, Col1, and β-actin as a loading control. The bands were quantified by ImageJ and the ratios are presented in panel C showing fold change in IPF lungs compared to control lungs. D. Control (C) or bleomycin (BL) treated mice ($n = 3$ mice/group) were euthanized, VCAM-1 protein levels in whole lung were detected by immunoblotting (inset) and bands quantitated densitometrically (below).

specimens from four IPF subjects and four control lungs. In control lung, VCAM-1 was poorly, if at all, detected in distal lung tissue (Fig. 3A). Importantly, VCAM-1 protein levels were markedly higher in IPF lungs with predominant localization in fibrotic areas (Fig. 3A, arrows) with additional positive staining detected in lung vessels (Fig. 3B, right panel) highlighting that lung fibroblasts are one major site of VCAM-1 expression in IPF lungs. To confirm our findings, we examined primary human fibroblasts isolated from IPF and control lungs. As seen in Fig. 4A and B, IPF fibroblasts had higher VCAM-1 protein compared to cells from control lung donors. Of note, treatment of control lung fibroblasts with TGF-β1, a crucial mediator of IPF pathogenesis increased VCAM-1 protein levels 4-fold in lung fibroblasts (Fig. 4C, D) suggesting that fibroblast-responsive up-regulation of VCAM-1 in IPF lungs may be caused by TGF-β1. To determine whether the increased VCAM-1 protein level is indeed induced by mRNA translation, total RNA was extracted from cells and real time PCR was performed. TGF-β1 triggered a several-fold increase in the VCAM-1 transcript that was maximal at 6 h of analysis (Fig. 4E). The response of VCAM-1 mRNA was still robust at 12 and 24 h of analysis after TGF-β1 exposure but was of lower magnitude. Additional analysis of VCAM-1 mRNA stability were conducted

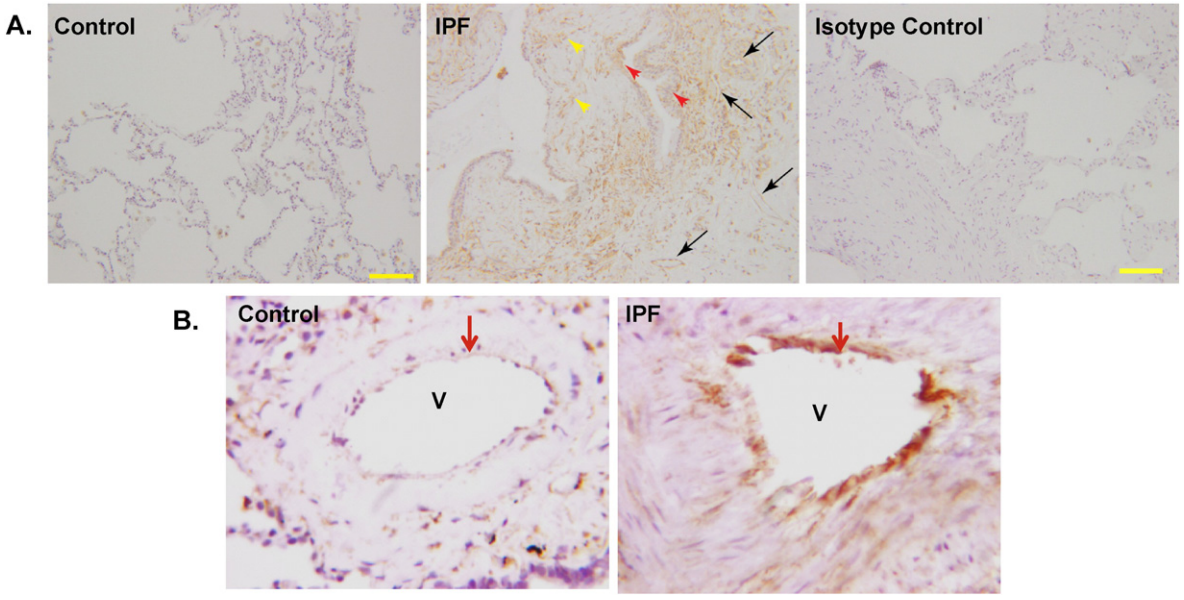

Fig. 3. Expression of VCAM-1 protein in IPF lung. A. Paraffin embedded IPF and control lungs were incubated overnight in primary antibody at 4 °C. An appropriate secondary antibody was used to identify the target protein on the lung tissue slides. The IPF lung show staining in endothelial cells (black arrows), airway epithelial cells (red arrowheads) and cells in the interstitium (yellow arrowheads). No staining was detected with the non-immune isotype control (magnification ×100, yellow inset bar = 100 μm). B. Brown staining for VCAM-1 protein in IPF lung vessels (V) (arrow) was detected and lack of staining was observed in control lung vessels.

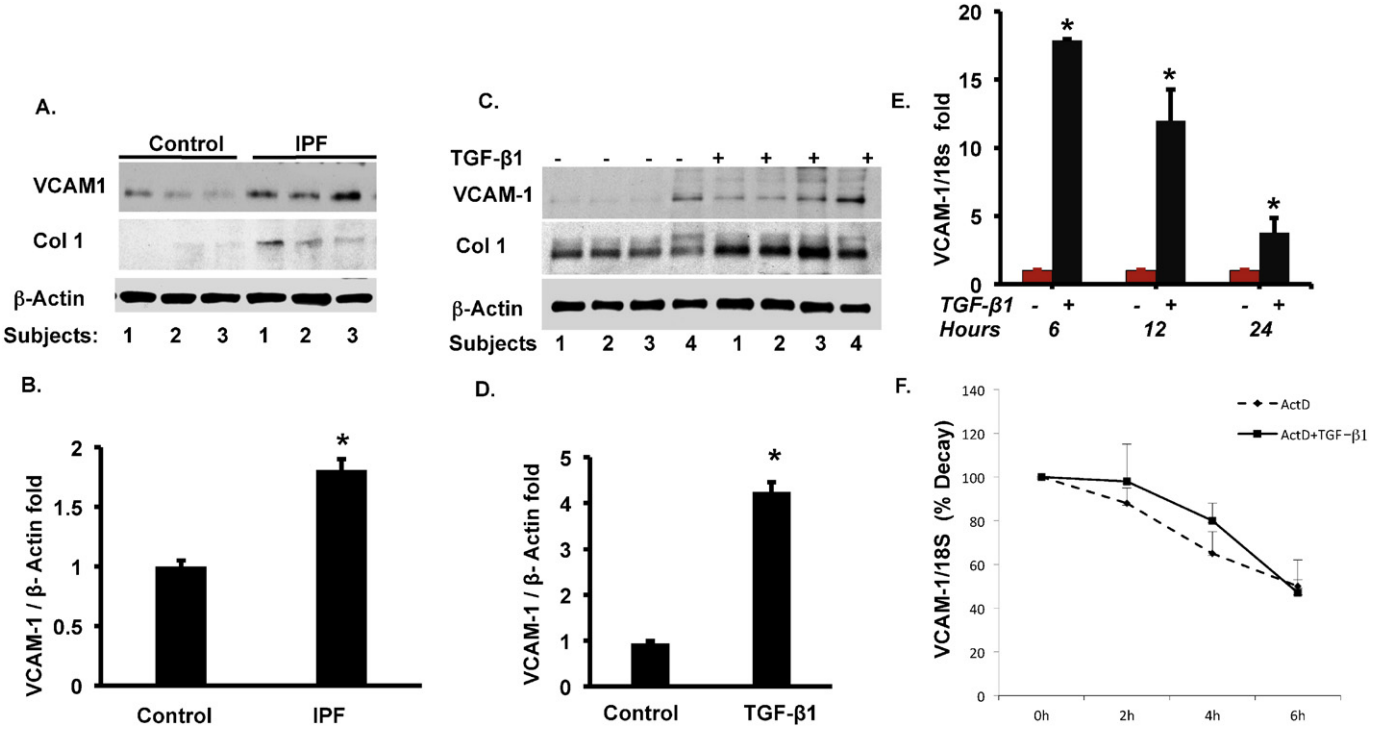

Fig. 4. Expression of VCAM-1 protein in primary lung fibroblasts. A–B. Control and IPF human lung fibroblasts were cultured and harvested at 70–80% confluence. VCAM-1 protein was examined by immunoblotting. Collagen 1 served as positive control for IPF lung fibroblasts and β-actin was used as a loading control of individual samples. The immunoblot shown is a representative of three independent experiments. In (B) individual bands were analyzed densitometrically and corrected for loading and densitometric ratios are shown as a bar graph. *$P < 0.05$ for control vs IPF groups. C–D. Lung fibroblasts were cultured and stimulated with TGF-β1 (5 ng/ml) for 24 h. The bands on immunoblots were quantified by ImageJ and densitometric ratios shown in Figure D. Statistical analysis was performed by using an unpaired student t-test. *$P = 0.0018$ for control vs TGF-β1 groups. Data shown are representatives of 3–5 independent experiments. E. Lung fibroblasts were cultured and stimulated with or without TGF-β1 (E) for 24 h. F. Lung fibroblasts were cultured in the presence of actinomycin D (ActD) with or without the inclusion of TGF-β1 (5 ng/ml) in the culture medium for various time points as shown. In both (E) and (F) total cellular RNA was extracted and real time PCR was performed to assay VCAM-1 mRNA as shown. The data represents three separate experiments except panel (F), $n = 2$.

using actinomycin D (Fig. 4F). VCAM-1 transcript exhibited a t ½ of ~6 h and the stability of the mRNA was not significantly altered by exogenous TGF-β1 administration. Taken together, these results suggest that TGF-β1 released in IPF lung robustly increases VCAM-1 gene transcription rather than modulating the lifespan of its transcript in human lung fibroblasts.

3.4. VCAM-1 regulates lung fibroblast proliferation

To investigate the physiologic role of VCAM-1 in human fibroblasts, we focused on cell proliferation given the role of TGF-β1 in cell growth and repair. Cells were transfected with a control shRNA or VCAM-1 shRNA and BrdU labeling were quantitated. Compared to lung fibroblasts treated with control shRNA, *VCAM-1* shRNA showed ~47% lower BrdU incorporation (Fig. 5A). We next assayed the effects of VCAM-1 depletion on cell cycle progression and observed that cells transfected with *VCAM-1* shRNA exhibited an increase in G0/G1 coupled to reduced G2/M and S-phase compared to control lung fibroblasts (Fig. 5B). To evaluate potential mechanisms of VCAM-1 depletion, we assayed immunoreactive levels of several mediators of proliferative signaling (Fig. 5C,D). Indeed, cells transfected with *VCAM-1* shRNA showed reduced levels of phosphorylated p38, extracellular signal-regulated kinase ½ (ERK ½) (Fig. 5C) and reduced mass of cyclin D1 (Fig. 5D). The results suggest that VCAM-1 abundance modulates specific regulatory components involved in fibroblast growth.

4. Discussion

The mechanisms whereby VCAM-1 levels are elevated in IPF and how this adhesion molecule might contribute to the pathobiology of disease are not well understood. The new findings from this study include (i) the demonstration of a negative correlation between lung VCAM-1 transcripts and pulmonary function, (ii) the identification that VCAM-1 protein expression is elevated in fibroblasts isolated from human IPF lungs, (iii) that VCAM-1 protein mass is increased in response to a pro-fibrotic regulator, TGF-β1 through a mechanism that is independent of effects on VCAM-1 mRNA stability, and (iv) that silencing *VCAM-1* expression inhibits fibroblast proliferation by inducing G_0/G_1 cell cycle arrest. These observations provide a biologic framework for future studies investigating the molecular regulation of this adhesion molecule at the level of gene transcription in IPF models.

Our previous work indicated that up-regulated VCAM-1 protein levels in the peripheral blood of IPF subjects are associated with increased mortality and is in line with the association of this biomolecule with disease risk, outcome, and severity [12]. However, to date, the source of VCAM-1 in the circulation was unknown in IPF subjects. We found that in addition to peripheral blood, VCAM-1 mRNA is also significantly upregulated in lung tissues from IPF patients compared to a control cohort. Given that IPF subjects may have pulmonary vascular hypertension, our data are consistent with prior observations showing that serum VCAM-1 also predicts the risk for vascular disease [23]. Interestingly, studies in systemic sclerosis report that increased VCAM-1 in serum is also associated with pulmonary involvement and pulmonary hypertension thus supporting a potential mechanistic role for this protein [24,25]. VCAM-1 is also observed in atopic asthma where it is related to disease activity [26]. While our data show significant differences in VCAM-1 protein and mRNA expression between IPF subjects and control subjects, we are aware of the limitations associated with a relatively small sample size for the group of subjects tested for validation of microarray datasets with IPF and control ($n = 11$/group). Despite this shortcoming, we were able to show consistently that an increase in IPF VCAM-1 protein in plasma using the existing cohort was sufficient

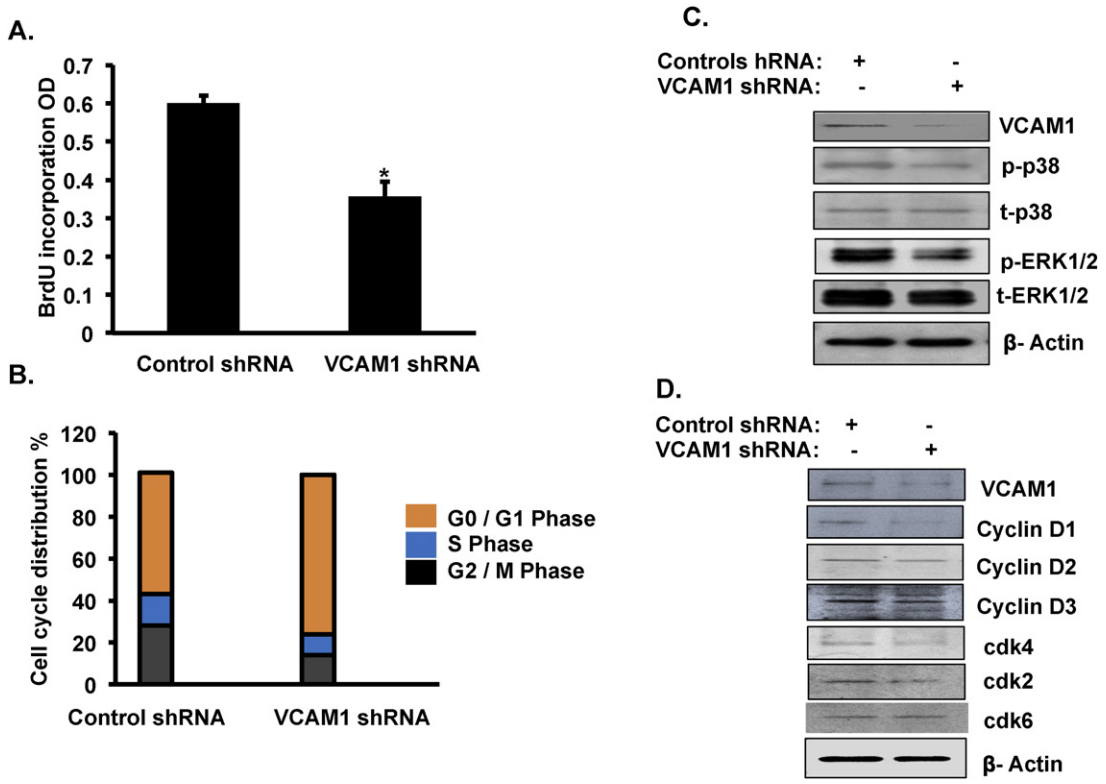

Fig. 5. VCAM-1 cellular depletion decreases fibroblast proliferation by impairing cell cycle progression. A. Human lung fibroblasts were transfected with control shRNA or *VCAM-1* shRNA and proliferation of cells was assessed using BrdU labeling as described in the Materials and methods section. Significantly reduced BrdU incorporation was observed in *VCAM-1* shRNA transfected cells (*$P = < 0.05$ in shRNA control vs *VCAM-1* shRNA). B. *VCAM-1* cellular depletion was conducted as in (A). *VCAM*-1 silencing on cell cycle progression was then determined in fibroblasts after depleting the adhesion molecule. Significantly reduced S phase and G2/M phase were observed in *VCAM-1* shRNA transfected cells ($P = < 0.05$) versus control shRNA. C-D. Human lung fibroblasts were cultured, transfected and harvested at 70–80% confluence as described above using shRNA. Cell lysates were harvested and processed for immunoblot analysis of indicated cell signaling proteins and key proteins involved in cell cycle regulation. The data are representative of $n = 2$ separate experiments.

to detect significant differences in VCAM-1 lung levels using immunoblot analysis. Given that lung mRNA VCAM-1 levels in IPF subjects negatively correlate with pulmonary function and VCAM-1 is shed during inflammation [27], these data suggest that the lung is one potential reservoir of blood-derived VCAM-1 that may be predictive of disease severity.

Because the cellular origin of VCAM-1 in pulmonary tissue may be linked to IPF disease pathogenesis, we further identified that VCAM-1 protein is expressed predominantly by IPF fibroblasts in fibrotic foci and is sensitive to TGF-β1 stimulation. Because TGF-β1 is a well-recognized driver of fibrotic lung remodeling, it is possible that increased VCAM-1 protein or mRNA concentrations in the peripheral blood may serve as an indicator of active fibrotic lung remodeling. Interestingly, high level expression of VCAM-1 in mouse lungs was observed in a murine model of radiation-induced pulmonary fibrosis, and its expression was significantly reduced after pulmonary manganese superoxide dismutase treatment, suggesting that at least in experimental chronic lung injury VCAM-1 levels might serve as an additional biomarker to monitor effects of anti-oxidant therapeutic intervention [28].

Not surprisingly, in addition to fibrotic foci and early-passage lung IPF fibroblasts, we found increased VCAM-1 levels in lung vessels in human IPF lung. Up-regulation of VCAM-1 is a sensitive marker of endothelial responses to oxidative stress. Further, vascular pathology is observed in IPF subjects with pulmonary hypertension [29,30]. While a mechanistic role of VCAM-1 in IPF is not yet fully elucidated, its role as a signaling molecule in other cell types is well documented [31,32]. As an example, hypoxic exposure up-regulates VCAM-1 together with other adhesion molecules, triggering an array of pro-inflammatory signaling events in pulmonary arteries [33]. Many of these signaling events are reversed during normoxia, but may be accentuated by mediators expressed in IPF. In this regard, TGF-β1, an integral mediator of pulmonary fibrosis, displays cross-talk with VCAM-1 raising a potential signaling mechanism that may be relevant in human IPF linked to increased adhesion molecules. The link between VCAM1 expression and TGF-β1, identified by us in IPF fibroblasts, raises the possibility that TGFβ1 may be a driving stimulus that alters VCAM-1 expression leading to both fibrosis and vascular pathology [34].

VCAM-1 transcripts are known to be upregulated by pro-inflammatory stimuli [35,36]. Here we observed a robust increase in both VCAM-1 mRNA and protein levels in response to exogenous TGF-β1 in human fibroblasts. The VCAM-1 mRNA lifespan was relatively short (t ½ ~ 6 h) consistent with the half-life of the transcript in synovial fibroblasts [35]. Interestingly, unlike the effects of IL-4 and TNF-α that induce VCAM-1 mRNA by stabilizing its transcripts, our data suggest that TGF-β1 triggers an increase in mRNA synthesis in human lung fibroblasts [35]. Our data also contrasts with findings of others in astrocytes where TGF-β1 inhibits TNF-α and IL-1β induction of VCAM-1 mRNA [36]. The ability of TGF-β1 that is centrally involved in IPF pathogenesis to elicit adhesion molecule expression in our studies also raises additional questions as to the physiological role of transcriptionally activated VCAM-1 on modulating fibroblast phenotypic behavior. We found that depletion of endogenous *VCAM-1* results in a decrease in fibroblast growth and reduction in levels of key signaling molecules implicated in cell survival and cycle progression. These results are consistent with observations elsewhere showing that VCAM-1 ligation activates ERK 2 resulting in increased expression of cyclin D1 [37]. Whether VCAM-1 is a bona fide molecular target for therapeutic intervention will require additional studies in preclinical models. These results do not exclude small molecule VCAM-1 inhibitors or biologics against adhesion molecules. In fact, studies are underway targeting the VCAM-1 co-receptor, α4β1 integrin,

in neoplasia supporting a role of this molecular apparatus in activating cell survival pathways [38]. However, given its promiscuous roles in numerous biological processes, the present data together with findings of others suggest a potential role of VCAM-1 as a biomarker in IPF and an upstream signaling molecule that regulates the fibroblast phenotype rather than a pharmaceutical target.

Conflicts of interest

None.

Portion of these findings have been presented at the American Thoracic Society conference, San Diego, May 2014.

Author Contributions

Conceived and designed the experiments: RKM, MA, LJV, NK, and EAG. Performed the experiments: LJV, and MA. Analyzed the data: LJV, MA, JRTand RKM. Contributed reagents/materials/analysis tools: LJV, DJK, and JS. Wrote the paper: MA, LJV, EAG, and RKM. Proofed paper and contributed to text: MA, LJV, NK, DJK, EAG, and RKM. Developed the IHC imaging, analyzed these data, and provided text material describing experiments: MA, LJV, EAG, DJK and JRT. Recruited subjects and organized specimen and clinical data collections, proofed paper and contributed some ideas/manuscript text: DJK and YZ. Edited paper and contributed scientific scrutiny NK, EAG, and RKM. Contributed to data treatment, statistical analyses and scientific proofreading; JRT.

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