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RESEARCH ARTICLE

CURCUMIN INHIBITS MMP-9 MEDIATED ANGIOGENESIS IN DIFFUSE PARENCHYMAL LUNG DISEASE

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF), a form of diffuse parenchymal lung disease (DPLD), is marked by honeycombing radiologically and characterized by dysregulated fibrogenesis. The other features include aberrant extracellular matrix (ECM) remodelling and angiogenesis. Hence, the aims of study were to evaluate whether MMP-mediated pathway is involved in angiogenesis and the effect of curcumin thereon in DPLD. Firstly, MMP-9 activity in broncho-alveolar lavage fluid (BALF) from with or without honeycombing patients was investigated by Zymography technique. Then BALF samples were evaluated for TNF-α and MMP-9 levels by Western blot analysis, while tubulogenesis by matrigel and angiogenesis by chick chorioallantoic membrane assays. Our study demonstrates that MMP-9 protein expression and activity levels associated with TNF-α protein expression were higher in the BALF of honeycombing +ve patients in comparison to the honeycombing –ve group suggesting MMP-9 plays a pivotal role in TNF-α-mediated down-stream axis protein expressions followed by tubulogenesis and vascularization for angiogenesis to occur. Incubation of BALF from honeycombing +ve patients with curcumin inhibited MMP-9 activity, and expressions of MMP-9, VEGF, pP38MAPK and Egr-3 molecules associated to angiogenesis. Therefore, Curcumin might have a therapeutic significance for arresting dysregulated angiogenesis in honeycombing +ve DPLD patients.

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INTRODUCTION

Patients with interstitial lung disease or DPLD usually show a chronic progressive course. A subset of such patients shows predominant pattern known as usual interstitial pattern (UIP). UIP pattern can be picked up well on high resolution CT scan of chest and formed by several disease states. IPF is an idiopathic category of lung fibrosis with UIP pattern after exclusion of the morphological contenders. The destruction of the alveolar architecture leads to progressive decline in pulmonary function in IPF (Mei *et al*., 2022). The scarred and stiffened lungs in IPF make it increasingly difficult to for gaseous exchanges in the lungs (Mandal, 2024) resulting in progressive shortness of breath, decreased exercise capacity, chest pain, dry cough and ultimately premature death (Fioret *et al*., 2011). The median survival time of IPF patients is approximately three years from the time of diagnosis (Hadi *et al*., 2023), however, predicting survival in patients with honeycombing has not been formerly evaluated. Incidentally, IPF has been regarded as a representative prototype of DPLDs with predominant fibrosis believed to result from an inflammatory response to an unknown substance (Margaritopoulos *et al*., 2012) leading to oxidative stress. The use of corticosteroid with or without chemotherapeutic agents and N-acetyl cysteine is no longer recommended (Raghu *et al*., 2011) and some molecules as pirfenidone and nintedanib are available as therapeutic agents to treat IPF (Glass *et al*., 2022). Though these drugs can ameliorate the disease state of IPF, they cannot cure the diseases, persisted with some adverse effects (Mandal, 2024). The very shifting of the concept of pathogenesis of IPF from inflammation induced fibrosis to dysregulated fibrogenesis has led to the introduction and trials with newer molecules with more hope but actual success (Raghu *et al*., 2011; Antoniou *et al*., 2006; Wells, 2006). This signifies that the precise cellular and molecular mechanism for fibroblast recruitment, proliferation, and lying of collagen in IPF or DPLD at large needs further elucidation. Therefore, the etiology and precise cellular and molecular mechanisms involved in IPF are yet to be elucidated (Koudstaal and Wijsenbeek, 2023; Rogliani *et al*., 2008). Curcumin, a polyphenol derived from the plant Curcuma longa, is a potent antioxidant, anti- inflammatory and anti-carcinogenic agent.

It acts on a variety of signal transduction pathways and molecular targets (Shahbaz *et al*., 2023; Ghosh *et al*., 2012) and may be effective for the treatment of DPLD patients. Angiogenesis, implicated in the pathogenesis of fibrotic disorders of the lungs, including IPF (Atkinson and Senior, 2003) is the process whereby formation of new blood vessel from pre-existing form may contribute to fibroproliferation and extra cellular matrix (ECM) deposition. Recently, the potential role of a class of zinc-requiring endopeptidases known as MMPs has been studied in the pathogenesis of IPF (Yue *et al*., 2021). Generally, they are secreted in latent proenzyme form and involved in the remodelling and degradation of ECM. An up-regulation of MMP-9, expressed mainly by macrophages and neutrophils and also by alveolar epithelial cells and fibroblasts (Pardo *et al*., 2016), can lead to release of growth factors from fibroblasts (Davies and Richeldi, 2002). Vascular endothelial growth factor (VEGF) has been recognized as a major regulator of pathological angiogenesis, enhancer of vascular permeability, and inducer of other potentiators of angiogenesis (Ando *et al*., 2010). In this regard, the inhibition of MMP-9 can be a potential therapy for IPF (Fujita *et al*., 2011).

Angiogenesis is distinct from vasculogenesis, which is the de novo formation of endothelial cells from mesoderm cell precursors (Risau and Flamme, 1995). The first vessels form through vasculogenesis, after which angiogenesis is responsible for most of the blood vessel growth during development and in disease (Flamme *et al*., 1997). Binding of VEGF to VEGF receptor-2 (VEGFR2) initiates a tyrosine kinase signaling cascade that stimulates vessel permeability, proliferation, migration and finally differentiation into mature blood vessels. Previous *in vitro* studies referred that the capillary endothelial cells could proliferate and exhibit tube structure upon stimulation by VEGF (Goto *et al*., 1993). Up-regulation of VEGF is a major component of the physiological response and its angiogenic property is considered as a possible treatment in vascular injury (Gavin *et al*., 2004). *In vitro* studies demonstrate that presence of VEGF causes endothelial cells proliferation, migration and forming tube that resemble capillary structures (Prior *et al*., 2004). Additionally, VEGF up- regulation occurs as a result of increased blood flow to affected areas which in turn increase the mRNA production of VEGFR1 and VEGFR2 and finally angiogenesis. Akt (protein kinase B), a homologue of the transforming viral oncogene v-akt, is activated by phosphotidylinositol (3,4,5)-triphosphate generated by activated phosphatidylinositol-3 kinase (PI3K) and is involved in angiogenesis (Paul *et al*., 2012). Moreover, P38 MAP kinases, a class of mitogen–activated protein kinases, are responsive to stress stimuli and participate in VEGF mediated cellular signaling. On the other hand, TNF-α is produced chiefly by activated macrophages and partly by many other cell types such as $CD4^+$ lymphocytes, NK cells and neurones which is able to induce inflammatory mediator, VEGF (Guruvayoorappan and Kuttan, 2008).

The aims of the present study were to systematically evaluate the activation and expression of MMP-9 in BALF from honeycombing +ve and -ve DPLD patients and to determine the relationship of MMP-9 levels with potentiator molecules involved in the cellular signaling for angiogenesis and inhibition of them by curcumin. We hypothesize MMP-9 mediated angiogenesis as major signaling pathway in DPLD and inhibiting role of curcumin thereon.

METHODS

Collection of BALF: The patients of DPLD from in and around Kolkata, West Bengal, were included in the study upon the availability of the written informed consents. They were diagnosed to have DPLD based on evaluation following the algorithm practiced at the institute with chest x-ray (postero-anterior view), followed by HRCT of chest and spirometry in clinically suspected cases. The patients with history of exacerbation in the preceding one month or having co-morbidities as significant liver or renal function impairment and congestive cardiac failure were excluded. Those who qualified were further divided into two groups based on the presence of honeycombing in their HRCT chest as patients with or without honeycombing. Subsequently, BALF containing cellular components was collected from the lower lobes of the lungs from patients with or without honey combing through performing fieroptic bronchoscopy following standard methods (Honeybourne *et al*., 2001). The details of the patients are listed in Table 1.

Table 1. The details of the honeycombing patients

	With honeycombing	Without honeycombing
Number	8	8
Male:Female	4:4	5:3
Mean age	57±11 years	66±15 years
Predominant HRCT findings	Honey combing	Ground glass opacity ± reticulation
Comorbidities	Dyspepsia (n=1), Hypertension (n=1), Hypothyroid (n=1)	Hypertension (n=1), Hypothyroid (n=1)
FVC (forced vital capacity) in absolute value and % predicted	1.91±0.45L,75±29% (n=4)*	1.24±0.37L, 49±15% (n=5)*

*based on the number of the available lung function records.
*since the primary objective of incorporation of the patients did not include spirometry, hence in the real world practice, the PFTs, though recommended, were not done by the participants.

Cellular BALF protein was estimated by Braford ELISA plate method and analyzed for MMP-9 enzymatic activity and protein expression of soluble mediators involved in angiogenesis. All the humans used in this study received proper care and handling in compliance with Human Ethics Committee, Registration No. ECR/159/Inst/WB/2013 issued under the rule 122DD of the Drugs and Cosmetics Rules 1945, India and only after receiving the approval of the Institutional (Institute of Pulmocare and Research, Kolkata, India) human ethics committee. All participants gave written informed consent for participation and the study protocols were approved by the Ethical Review Boards of the concerned Institute.

Gelatin Zymography: For the MMP-9 assay, BALF (6 µg protein) from each patient was electrophoresed in 8% resolving SDS-polyacrylamide gel containing 1.2 mg/mL of gelatin, pH 8.8 under non- reducing conditions. The gels were washed twice in 2.5% Triton-X-100 and incubated for 18 h at 37ºC with calcium assay buffer (CAB) (40 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10mM $CaCl_2$) for gelatinases. The gels were stained with 0.1% coomassie blue followed by destaining. The zones of gelatinolytic activities indicative of zymographic bands were quantified using densitometry linked to proper software, Lab Image (Kapelan Gmbh, Germany).

Western Blot: BALF samples (24 µg protein) collected from each patient were resolved by 8% SDS- polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After transfer, the membranes were blocked with 3% BSA solution in 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 0.02% Tween 20 (TBST) and followed by overnight incubation at 4ºC in 1:500/400 dilutions of the respective primary antibodies (Santa Cruz Biotechnology) in TBST containing 2% BSA. Membranes were washed, incubated again with alkaline phosphatase-conjugated secondary antibodies (1:10000) (Santa Cruz Biotechnology) for 3 h and washed with TBST and TBS. Blots were developed using 5- bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium (BCIP/NBT) substrate solution (Sigma-Aldrich) and the fold changes activities expressed were analyzed with densitometry linked proper software (Lab Image, Kapelan Gmbh, Germany).

Assay of drug dose response with cell viability: The assay was determined in broncho-alveolar lavage cells treated with curcumin using the trypan blue exclusion method. This method was utilized to determine the number of viable cells present in cell suspension based upon the principle where live cells exist intact cell membranes excluding trypan blue, whereas dead cells having no capacity to exclude trypan blue. In brief, broncho-alveolar lavage cells were seeded at a density of $1x10^4$ cells / mL and incubated with different concentrations of curcumin (5µM, 15µM, 25µM, 35µM) at 37°C in a humidified atmosphere of 5% CO_2 in air for 2h. Cell viability was monitored microscopically with hemocytometer. The number of cells absorbed or excluded the dye were counted, from which the percentage of viable cells over total number was calculated. The viability assay by curcumin was assessed with respect to untreated control. Each measurement was performed in triplicate. The data denote mean values from four different BALF samples of honeycombing +ve patients.

Assays of different molecules and their inhibitions: BALFs (6 / 24 µg proteins) from honeycombing +ve DPLD patients (n=2, randomly selected) were incubated with various concentrations (0-25µM) of curcumin (purchased from MP Biomedicals) (dissolved in 0.2% Tween 20) for 1.5 h at 37ºC. After incubation the BALFs were electrophoresed in SDS-polyacrylamide gel for gelatin zymography and western blotting to quantify their expressions.

Tubulogenesis: To examine the effects of BALF samples on tubulogenesis, Ehrlich ascites carcinoma (EAC) cells ($1x10^4$/well) were incubated with BALF samples (24 µg) in 24-well culture plates that were pre-coated with matrigel (Cell Biolab, Inc). After 24 h of treatment, the representative images (10X) were obtained using fluorescence microscopy in order to assess tube formation and quantify branch number per field.

Angiogenesis: Day 0 fertilized eggs of white leghorn chickens were kept at 37ºC under sterile conditions. BALF sample (24 µg) impregnated carboxymethylcellulose discs were implanted after nine days of incubation in the chick chorioallantoic membrane (CAM) through a 1 cm^2 window made on the shells of different eggs. After resealing, the eggs were again incubated at 37ºC in a humidified chamber for 72 h. The eggs were then opened and photographed to perform quantitative measurements of the branching points and length of the vessels. The angiogenic index was determined by measuring the percentage of a unit area taken up by blood vessels.

Statistical analysis: Data were fitted using Sigma plot and are presented as mean ± SEM. Statistical analysis was performed using Student's t-test / Bonferroni-corrected two-way analysis of variance (ANOVA) in GraphPrism Instat 5 software.

RESULTS

Activity of MMP-9 in BALF of honeycombing +ve DPLD patients: BALF collected from honeycombing -ve and +ve patients (n=8 for each group) were assessed for MMP-9 activity. Figure 1P exhibits the positive association of gelatinolytic activity in BALF of honeycombing +ve patients in comparison to honeycombing -ve patients. The major gelatinolytic bands for the honeycombing +ve patients are significantly higher relative to the –ve group. Patients' BALF showed higher pro and active MMP-9 activities (C in contrast to A, and D in contrast to B) (average ~ 2.47 fold and ~ 2.08 fold respectively) (Figure 1Q).

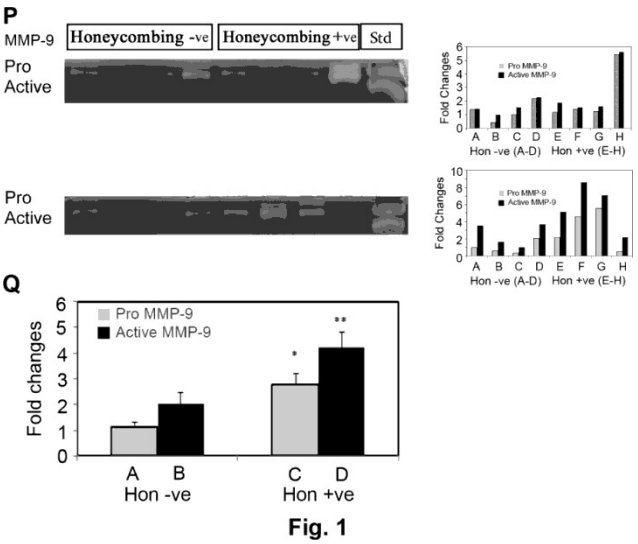

Figure 1: Activity of MMP-9 in BALF of honeycombing patients (+ve and –ve) (P and Q). Average fold changes in the activity of MMP-9 (Q). For zymography - A, Pro-MMP-9; B, Active-MMP-9; C, Pro-MMP-9; D, Active-MMP-9. Honeycombing -ve and +ve groups for one set contain 8 samples, 4 for each. Error bar; mean ± SEM obtained from three independent experiments. * p< 0.05, ** p<0.01 compared with their Pro-forms of MMP-9 and Active-forms of MMP-9 between honeycombing -ve and +ve groups

Expression of TNF-α and MMP-9 in BALF of honeycombing patients: To evaluate expressions of TNF-α and MMP-9 in BALF of honeycombing +ve patients, we conducted Western blot analysis. Figure 2 shows that TNF-α protein expression in the BALF of honeycombing +ve patients is ~ 2.8 fold higher than the –ve group (n=4 for each group of randomly selection) and is statistically significant. The expression of MMP-9 was higher in BALF of honeycombing +ve patients relative to the -ve group (n=4 per group of randomly selection) where respective change was ~ 2.3 fold higher.

BALF of honeycombing patients promoted angiogenesis in matrigel and CAM assays: The angiogenic potential of BALF was substantiated by a matrigel assay (Figures 3 and 5P). Our results strongly suggest that application of BALF of honeycombing +ve patients increased tube formation and branch number in contrast to honeycombing -ve patients.

However, BALF of honeycombing -ve and +ve patients showed potent tubulogenesis with maximum tube formation observed at a change of ~1.7 and ~3.4 folds higher respectively compared to control.

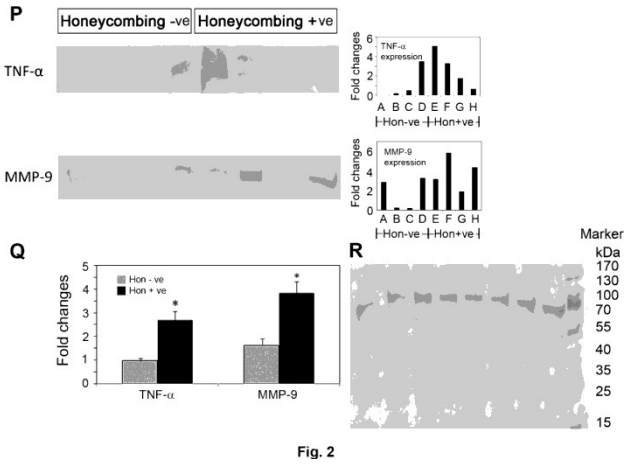

Fig. 2

Figure 2. Increased expressions (P) and average fold changes (Q) of TNF-α and MMP-9 in BALF of honeycombing +ve compared to –ve samples. Error bar; mean ± SEM obtained from three independent experiments. * p< 0.01 for honeycombing +ve in comparison to –ve. Equal loading of BALF protein in each lane was visualized through Ponseu S staining of the blot (R).

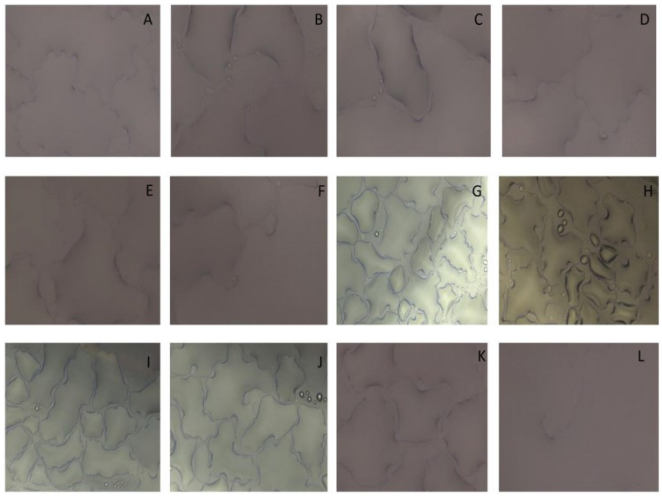

Fig. 3

Figure 3. The honeycombing +ve samples (G, H, I, J, K) (n=5) indicated the maximum increased tube formations in BALF-treated Ehrlich ascites carcinoma (EAC) cells cultured in matrigel coated 24-well plates in contrast to honeycombing -ve samples (B, C, D, E, F) (n=5). Controls (A, L) (n=2) indicate less tubes.

To complement the investigation of the angiogenic potential of BALF in honeycombing +ve patients, we performed an angiogenic assay using a chick chorioallantoic membrane (CAM) model where BALF of honeycombing +ve patients developed more capillaries and branching points than honeycombing -ve patients (Figure 4). Implantation of a BALF-impregnated disc (80 µg) resulted in approximately ~2.9 and ~1.9 fold and ~5.7 and ~4.9 fold increase in the blood vessel length and branching points in honeycombing -ve and +ve patients respectively, when compared to those in vehicle-impregnated vessels (control) (Figure 5Q).

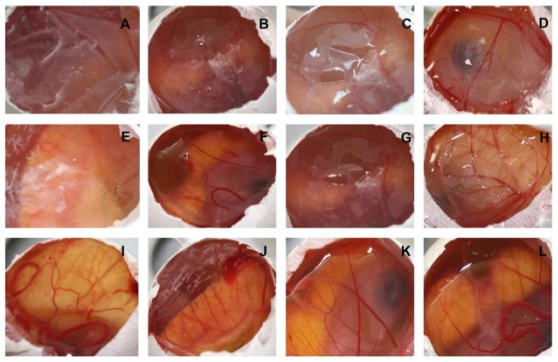

Fig. 4

Figure 4. The honeycombing +ve fluids (H,I,J,K,L) (n=5) exposed on CAM model showed maximum vascularizations for capillary formations in angiogenesis compared to honeycombing -ve fluids (C,D,E,F,G) (n=5) and minimum in controls (A and B) (n=2) (PBS exposed)

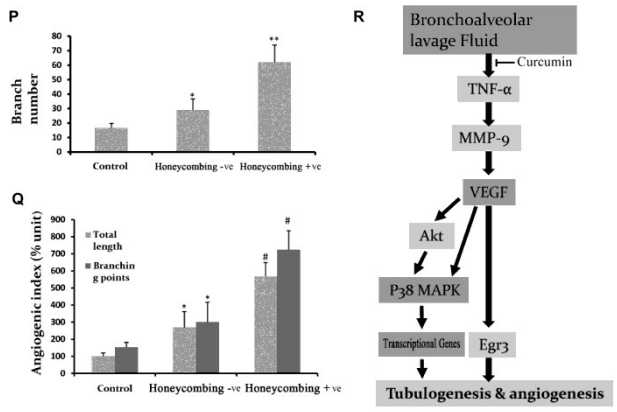

Fig. 5

Figure 5: Histographic representations of number of branches (P) and angiogenic index (Q) of vehicle (control), honeycombing -ve and +ve BALFs exposed / implanted in matrigel / CAM assays for tubulogenesis and vasculogenesis experiments. All of these results are representative of three different experiments. Error bar; mean ± SEM. * p< 0.05 compared with vehicle (Control). ** / # p < 0.001 compared with honeycombing -ve. Probable schemetic diagram of angiogenic pathway and its inhibition in honeycombing +ve BALF samples (R).

Cytotoxicity: The effect of curcumin on broncho-alveolar lavage cells incubated for 2h was evaluated. The cytotoxicity of curcumin on broncho-alveolar lavage cells was noticed to be dose dependent (EC50, ~30µM) (Figure 6) demonstrated by gradual reduction in cell number from ~100% to ~46% exposed with 0-35µM curcumin.

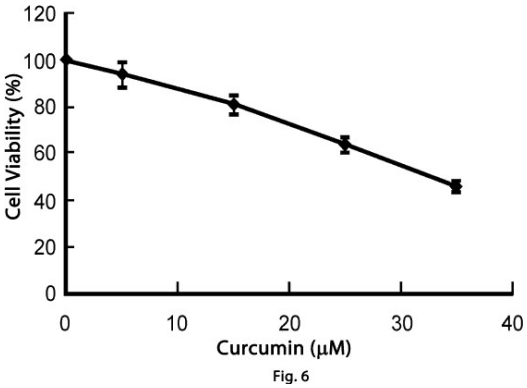

Fig. 6

Figure 6: Curcumin depicted cytotoxicity toward broncho-alveolar lavage cells in a dose dependent manner. The viability of untreated broncho-alveolar lavage cells (control) was defined as 100%

Expressions of MMP-9 and other molecules in the BALF of honeycombing +ve DPLD patients and the effect of curcumin treatment: The average level of MMP-9 activity in the BALF of honeycombing +ve DPLD patients was found significantly higher (~5 fold) compared to the control while curcumin incubation reduced this activity ~ 3 fold lower in comparison to honeycombing +ve value. The average expressions of MMP-9, VEGF, pP38MAPK and Egr 3 were noticed~ 2,~4,~3.5 and~4.5 folds higher significantly compared to controls respectively while curcumin incubation decreased~ 2,~ 2.5,~ 2.8,~ 4.4 folds lower expressions in contrast to honeycombing +ve values respectively. Curcumin also exhibited a significant dose-dependent reduction in the *in vitro* MMP-9 activity and expression of all molecules in BALF (Figure 7Q).

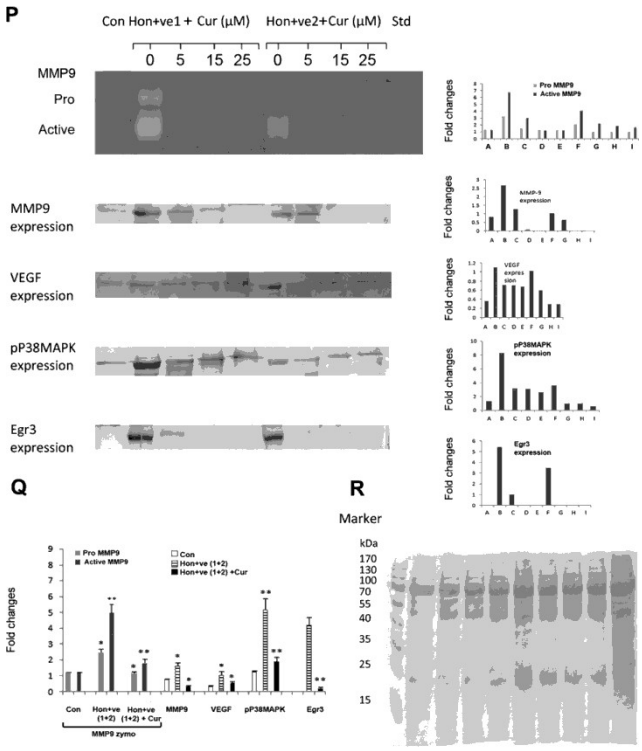

Fig. 7

Figure 7: Inhibitory role of curcumin on activity of MMP-9 and over expressions of MMP-9, VEGF, pP38MAPK and Egr3 in vitro. Control BALF samples were collected from the unaffected lungs from patients. Equal amount of BALF protein from patients with honeycombing +ve was incubated with different concentrations of curcumin and subjected to gelatin zymography (P). Histogram (P) represents MMP-9 activity and expressions of molecules with fold changes where A, control; B, hon+ve 1; C, hon+ve 1 + 5µM cur; D, hon+ve 1 + 15µM cur, E, hon+ve 1 + 25µM cur; F, hon+ve 2; G, hon+ve 2 + 5µM cur; H, hon+ve 2 + 15µM cur; I, hon+ve 2 + 25µM cur treatments. Histogram (Q) represents average fold changes from three independent experiments. *P<0.01 and **P<0.001 compared with control / honeycombing + ve (1+2). Equal loading of BALF protein in each lane was visualized through Ponseu S staining of the blot (R).

DISCUSSION

MMPs, capable of degrading various components of connective tissue matrices, are believed to play a significant role in matrix remodeling following parenchymal damage through induction of repair mechanisms in lung disease (Suga *et al*., 2000; Henry *et al*., 2002). In the present study, it has been demonstrated that MMP-9 was predominantly enhanced in the BALF of honeycombing +ve patients which was more than 2.08 fold higher in activity and protein expression as reflected by gelatin Zymography analysis and Western blot techniques respectively. To the best of our knowledge, this is the first study that investigated the role of TNF-α, MMP-9, VEGF, p-P38MAPK and Egr-3 in the pathogenesis of DPLD in honeycombing +ve patients. We used the BALF, a minimally invasive tool, in order to evaluate the activity of MMP-9 as well as protein expression of relevant angiogenic molecules. TNF-α is implicated in orchestrating inflammatory cell accumulation during pathological angiogenesis (Henry *et al*., 2002). In our study, the BALF of honeycombing +ve patients, showed more TNF-α release compared to the -ve group in terms of protein expression which is associated with pulmonary inflammation and promotion of angiogenesis by the enhanced activation and expression of MMP-9 (Guruvayoorappan and Kuttan, 2008; Xavier *et al*., 2010). It is noteworthy that anti-TNF-α-directed therapy has also been suggested as a possible treatment for DPLD (Ganguly and Swarnakar, 2009). In IPF, it has been recognized that the structural integrity of the alveolar epithelial cell wall depends on the basement membrane and that destruction of the sub-epithelial basement membrane may precede the development of alveolar fibrosis (Hecker and Thannickal, 2011).

A discontinuity of the basement membrane allows for greater access of exudative factors and interstitial cells to the alveolar space, potentially promoting further tissue destruction and progressive fibrosis (Suga *et al*., 2000). VEGF is known to promote both neo-angiogenesis and increase vascular permeability, in addition to epithelial proliferation (Rak *et al*., 1995) thereby supporting a role for MMP-9 induced-VEGF expression. Moreover, MMP-9 can activate matrix bound growth factors such as VEGF (Hawinkels *et al*., 2008) which increase the bioavailability of later and may be of clinical significance. In the present study, we found increased VEGF expression in the BALF of honeycombing +ve patients relative to the -ve group and that VEGF may activate downstream mediators such as Akt, which in turn initiate angiogenesis.

Additionally, it is well established that angiogenesis is directly modulated by mitogen- activated protein kinases (MAPKs). Therefore, MAPK activation was determined in BALF of honeycombing +ve and -ve patients. P38MAPK showed higher levels of activation as demonstrated by increased pP38MAPK protein expression in the BALF of honeycombing +ve patients in comparison to the –ve group. Together our data suggest that Akt and MAPKs may play significant role in promoting angiogenesis. VEGF response element, early growth response-3 (Egr-3) is involved in the VEGF-triggered signaling pathways in endothelial cells (ECs). Upon binding to VEGFR2, VEGF activates several signaling pathways in ECs, leading to activation of serum response element (SRE), nuclear factor of activated T-cells (NFAT) and cAMP-responsive element (CRE) which may bind to the promoter elements of the Egr-3 gene specifically in BALF of honeycombing +ve patients to increase transcription levels of Egr-3, and execute VEGF-induced vascular functions such as tube formation, vascularization, and EC proliferation for the processing of angiogenesis. In order to unravel MMP-9 functions in angiogenesis, we performed tubulogenesis in vitro assays. Ehrlich ascites carcinoma cells were plated on a collagen matrix to form tubes.

The effect of BALF on tube maintenance was assessed by counting the number of branches per field, after 24 h of treatment. In these conditions, we observed that control EAC or non-IPF BALF-treated EAC exhibited less branching tubules, as expected (Figures 3 and 5P). Strikingly, in honeycombing +ve BALF-treated EAC, numerous tubular structures persisted, indicating a role for VEGF through activation of MMP-9 in *in-vitro* tube maintenance. Therefore, our tubulogenesis and vasculogenesis experiments confirmed that VEGF might activate P38MAPK and Egr-3 in EAC as well as in chick chorioallantoic membrane that treated with BALF of honeycombing +ve patients (Ralph *et al*., 2011; Sharma *et al*., 2012). In conclusion, the present study systematically assessed a mechanism for processing of angiogenesis in DPLD patients with or without honeycombing through BALF analysis where MMP-9 activity correlates with increased protein expression and in turn related to downstream sequence of events in signaling pathways (Figure 5R) (Mohankumar *et al*., 2015; Zhao *et al*., 2016; Jun-ichi *et al*., 2010). However, the unknown aetiology of IPF especially environmental factors including smoking (clinical history not maintained at the time of collection of BALF samples) in honeycombing patients may also influence angiogenesis in DPLD. The inhibitory effect of curcumin (Figure 7) against all the molecules such as MMP- 9, VEGF, p-P38MAPK and Egr-3 in the BALF of honeycombing +ve DPLD patients and a gradual decrease in the activity and expression of MMP-9 and other molecules in a dose dependent manner indicated its inhibitory role by deactivation mainly on upstream key molecule, MMP-9 in the progression of angiogenesis (Woo *et al*., 2005) where the lower dosages (5-25μM) of curcumin indicated its affectivity for inhibition of marker protein expressions in BALF of honeycombing +ve DPLD (Figures 6 and 7). In this study, we selected eight BALF samples per group (honeycombing +ve or –ve) to assess and differentiate primarily any role of MMP-9 activity (Figure 1) in DPLD. After getting more impact of the biological markers in honeycombing + ve BALF (Figure 1), we took four BALF samples from each of honeycombing +ve and –ve groups (Figure 2) to assess expressions of TNF-α and MMP9 involved in angiogenesis. As the progress of angiogenesis in honeycombing +ve group was prominent (Figure 2), we chose two BALF samples from honeycombing +ve group to verify the effect of curcumin (Figure 7). The activation and over-expression of MMP-9, due to tissue injury, chronic inflammation and fibro-proliferative disorders, may contribute to the formation of a microenvironment dissolving extracellular matrix and initiating and promoting angiogenesis through the expression of VEGF, an endothelial cell signal key protein and sprouting of new vessels from pre-existing vasculature and ultimately diffuse parenchymal lung disease characterized by abnormal activation of bronchial alveolar cells secreting various mediators such as MMP-9 involved in the recruitment and maintenance of altered fibroblast and the proliferation of myofibroblasts, excessive accumulation of extracellular matrix making the loss of lung architecture and developing fibrosis, cancer and metastases. Thus MMP-9 can degrade extracellular matrix as well as has capacity to release, cleave and activate cytokines, growth factors, chemokines and receptor proteins affecting normal cellular functions to diseased state. Therefore, the activation and over expression of MMP-9 may have a key link on the progression of diffuse parenchymal lung disease with angiogenesis (Pardo *et al*., 2016).

Therefore, it may be concluded that curcumin might be considered as a potent therapeutic agent to inhibit angiogenesis in honeycombing +ve DPLD patients.

Conflict of interest: None declared.

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