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HIF-1 α signaling by airway epithelial cell K α 1-tubulin: Role in fibrosis and chronic rejection of human lung allografts

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Abstract

Long term survival of the human lung allografts are hindered by chronic rejection, manifested clinically as bronchiolitis obliterans syndrome (BOS). We previously demonstrated significant correlation between the development of antibodies (Abs) to K α 1-tubulin (K α 1T) and BOS. In this study, we investigated the molecular basis for fibrinogenesis mediated by ligation of K α 1T expressed on airway epithelial cells by its specific Abs. Using RT-PCR we demonstrate that normal human bronchial epithelial (NHBE) cells upon ligation of K α 1T with specific Abs caused upregulation of pro-fibrotic growth factors. Western blot analysis of NHBE incubated with K α 1T Abs increased hypoxia inducible factor (HIF-1 α). K α 1T Ab-mediated growth factor expression is dependent on HIF-1 α as inhibition of HIF-1 α returned fibrotic growth factor expression to basal levels. In conclusion, we propose that HIF-1 α -mediated upregulation of fibrogenic growth factors induced by ligation of K α 1T Abs is critical for development of fibrosis leading to chronic rejection of lung allograft.

1. Introduction

Chronic rejection following human lung transplantation clinically manifested as bronchiolitis obliterans syndrome (BOS) continues to be deleterious for the long term survival of the allograft [1]. BOS is a fibroproliferative process that involves inflammation and progressive fibrosis of the lamina propria and luminal occlusion of the small airways resulting in progressive decline in pulmonary function and eventual graft failure. Previous studies from our laboratory, and others, have implicated that the development of antibodies (Abs) to donor HLA and non-HLA antigens (Ags) including K α 1Tubulin (K α 1T) and Collagen predisposes lung transplant recipients for the development of chronic rejection. [2-4]. Airway epithelial cells (AECs) are shown to be the major immunologic targets for the pathogenesis of lung allograft rejection [5-7]. It has been demonstrated that activated epithelial cells produce high levels of fibrotic growth factors, including EGF, heparin binding EGF, basic FGF, and TGF- β [6, 8]. Upregulation of these growth factors have been

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demonstrated during BOS development following human lung transplantation [9, 10]. However, the intracellular signaling mechanisms, as well as the stimuli for the production of fibrogenic growth factors during BOS development, are yet to be defined.

The HIF-1 α is a well-known nuclear transcription factor that binds specifically to hypoxia response element on the promoter region of various hypoxia-inducible genes which are known to be involved in angiogenesis, oxygen transport, growth factor signaling, and apoptosis [11]. HIF-1 α stimulates the expression of pro-fibrotic genes such as vascular endothelial growth factor (VEGF) [12, 13]. Using comparative expression profiling Tzouveleakis et al have demonstrated a potential role for HIF-1 α in the pathogenesis of pulmonary fibrosis [14]. Recently, using animal models Jiang et al have suggested a potential pro-angiogenic role of HIF-1 α and thereby attenuating rejection process [15]. It would be interesting to check the role of HIF-1 α in a complex transplant setting with apparently opposing role, *i.e.* pro-angiogenic role promoting transplant survival and pro-fibrotic role leading to transplant rejection.

Previous reports from our laboratory demonstrated that development of Abs to epithelial gap junction protein K α 1T are developed following human lung transplantation and correlated with the development of chronic rejection following human lung transplantation [16]. Furthermore, *in vitro* studies also demonstrated a role for lipid rafts in the K α 1T Abs mediated upregulation of pro-fibrogenesis in cultured primary bronchial AECs [17]. However, the mechanism of K α 1T Ab mediated fibrosis remains unknown. In this report, we demonstrate that ligation of K α 1T expressed on the AECs causes activation and induces the HIF-1 α dependent pathway leading to fibrogenic growth factor upregulation which is a hallmark of BOS and other airway constrictive diseases.

2. Materials and Methods

2.1. Cell culture

NHBE cells were obtained from the American Type Culture Collection (CRL-2503, ATCC, Manassas, VA) and cultured in small airway cell basal medium SAGM™ along with the supplement (catalogue No.: CC-3119 and CC-4124, Lonza, USA) provided by the company. Cell lines were frozen at -130°C until use. Upon thawing, cells were maintained in 5% CO₂ incubator in sterile growth media at 37°C. Cells were then stimulated with varying concentration of Abs to K α 1T (Santa Cruz Biotech, CA) for 5min, 10min and 15min. In parallel, for inhibition of individual components of MAP Kinase complex, cells were treated with the specific inhibitor of p38 SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole, Sigma Aldrich, St. Louis, MO) at concentrations of 0.1-100 μ M for 30min before the addition of K α 1T Abs. The same protocol was also carried out using MAP Kinase inhibitors of ERK 1/2 (U0126, Sigma Aldrich, St. Louis, MO) and JNK (SP600125, 1,9-Pyrazoloanthrone, Sigma Aldrich, MO) and a vehicle control (dimethyl sulfoxide at concentration of 0.1%). JNK inhibitor SP600125 was used at concentrations of 0.1-100 μ M, and ERK inhibitor U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate, Sigma Aldrich, St. Louis, MO) was used at 0.1-100 μ M [18]. For HIF-1 α inhibition, NHBE cells were incubated with YC-1, (3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole, Sigma Aldrich, St. Louis, MO) at concentrations of 0.1-100 μ M for 6hrs [19]. For evaluating HIF-1 α signaling, specific prolyl-4- hydroxylase (PHD) inhibitor (Sigma Aldrich, St. Louis, MO), NHBE cells were treated with dimethyl oxalylglycine N-(Methoxyoxoacetyl)-glycine methyl ester, (DMOG, Sigma Aldrich, St. Louis, MO) at final concentration of 1mM for 6hrs prior to activation with K α 1T Abs [20, 21]. All experiments were performed in triplicates.

2.2. Total protein and nuclear protein extraction

Total proteins were extracted from cells with lysis buffer (50mM HEPES [pH 7.6], 150mM NaCl, 1% Triton X-100, 30mM Na₄P₂O₇, 10% glycerol, 1mM benzamide, 1mM DTT, 10µg of leupeptin/ml, 1mM phenylmethylsulfonyl fluoride 50mM NaF, 1mM sodium orthovanadate, 10mM sodium pyrophosphate decahydrate, 10mM β-glycerophosphate). All chemicals for the buffer preparation have been obtained from Sigma Aldrich (St. Louis, MO). After cell lysis, the supernatant was collected and run at $15,000 \times g$ for 15min at 4°C. For nuclear protein extraction from NHBE cell culture, the cells are scrapped and with initial resuspension in cold PBS. The cells are pelleted by centrifuging for 5 min at 2000 rpm at 4°C. The pellet is resuspended in 25mM HEPES, 5 mM KCl, 0.5 mM MgCl₂, 1mM phenylmethylsulfonyl fluoride, 1 mM hithiothreitol, 1 ng/mL Aprotinin and 10 ng/mL Leupeptin in 0.1% NP-40 buffer at pH 7.6 and centrifuged at 2500 rpm for 3 min at 4°C. The supernatant (cytosolic extract) is saved separately. The pellet is washed in cold PBS and then resuspended in 25mM HEPES, 10% sucrose, 350 mM NaCl, 1mM phenylmethylsulfonyl fluoride, 1 mM hithiothreitol, 1 ng/mL Aprotinin and 10 ng/mL Leupeptin buffer at pH 7.2 and rotated for 1 hr at 4°C. The solution is then centrifuged at maximum speed for 10 min and supernatant containing nuclear extract is collected. Protein concentration was determined with a Bradford's assay kit from Bio-Rad (Munich, Germany).

2.3. Western blot analysis

The protein extracts were separated on a 4-12% gradient gel and electrophoretically transferred onto a nitrocellulose membrane. The cytoplasmic protein extracts were used for the analysis of mTOR, Akt-1, HIF-1α and nuclear protein extracts were used in the analysis of p38, ERK-1/2, c-JNK. The membranes were blocked overnight at 4°C and probed by appropriate primary and secondary Abs. All primary and secondary Abs were obtained from Santa Cruz Biotech (Santa Cruz, CA), along with catalogue no. provided with individual antibody. Active phosphorylated form of mTOR (sc8319, MW-211 kDa), Akt-1 (sc7126, MW-60 kDa), p38 (sc271120, MW-38 kDa), ERK-1/2 (sc135900, MW- 42 kDa and 44 kDa), and c-JNK (sc7345, MW- 54 kDa) were probed with phosphor-specific ser-2448/FRAP1-mTOR (sc101738), Thr-308/Akt1 (sc135650), Thr-180/Tyr-182/p38 (sc17852), Thr202/Tyr204/ERK-1/2 (sc136521), and Thr-183/Tyr185/c-JNK (sc81502), respectively. The membrane was developed using the chemiluminescence kit (Millipore) and analyzed on using Bio-Rad Universal Hood II (Hercules, CA). Densitometric analysis was done using the software provided by the company. The 'fold increase' of HIF-1α to quantitate the modulation of protein expression was calculated from the blot densitometry using the equation: density upon cross-linked Kα1T Abs treatment/density without cross-linked Kα1T Abs treatment. The 'fold increase' of transcription factors/proteins to quantitate the activation from phosphor-blot analysis was calculated based on densitometric analysis with the following equation: [(phosphorylated form upon Kα1T Ab treatment/unphosphorylated form upon Kα1T Ab treatment) × (unphosphorylated form non- Kα1T Ab treatment/ phosphorylated form non- Kα1T Abs treatment)].

2.4. Growth factor determination

Expression profiles of intracellular signalling genes in the isolated NHBE were analyzed using the FAM-labeled RT-PCR primers for VEGF, PDGF, bFGF and TGF-β (Hs00900055_m1, Hs00181813_m1, Hs00915142_m1 and Hs00998133_m1, respectively; Applied Biosystems, Foster City, CA) as per the manufacturer's recommendation. Briefly, total RNA was extracted from 10⁶ cells using TRIzol reagent (Sigma–Aldrich). RNA samples were quantified by absorbance at 260nm. The RNA was reverse-transcribed and RT-PCR (real time PCR) was performed in a final reaction volume of 50µL using iCycler 480 Probes Master (Roche Diagnostics). Each sample was analyzed in triplicate. Cycling

conditions consisted of an initial denaturation of 95°C for 15min, followed by 40 cycles of 95°C for 30s, followed by 61°C for 1min.

2.4. Statistical analysis

Data are expressed as mean $\pm$ SEM for a set of four different experiments. Statistical differences between means were analyzed using a paired or unpaired Student's t test. A *p*-value of less than 0.05 was considered significant. All data analysis was obtained using Origin 6 software (Origin Labs, Northampton, MA).

3. Results

3.1. Up-regulation of growth factor expression in AECs upon ligation of K α 1T with its specific Abs

We have previously reported that treatment of normal human bronchial epithelial (NHBE) cell cultures with sera from BOS(+) K α 1T(+) patients caused significant up-regulation of growth factor expression profile [16]. To test for the direct role of the K α 1T Abs in the induction of growth factors, we co-cultured NHBE cell cultures incubated with mAb to K α 1T. As shown in figure 1A, we titrated K α 1T Abs concentration from 0.1 to 100 μ g/mL and observed a dose dependent increase of VEGF mRNA expression. However, when cross linking was done using a secondary anti-mouse immunoglobulin VEGF transcript levels achieved plateau phase at near 0.5 μ g/ml of K α 1T Abs. Therefore, to analyze the effects of K α 1T Abs on NHBE for the remainder of the studies we employed crosslinking of the primary with Abs to mouse immunoglobulins. The need for cross-linking has been reported in various immunologic studies with T-cell receptors [22], B-cell receptors [23], and high affinity IgE receptor (Fc ϵ R1) [24]. The membrane lipid raft mediated signaling has been associated with this cross-linking [25] and might also help intercellular continuum. However, direct evidence has not been established using methods such as electron microscopy and x-Ray crystallography. In spite of this limitation, it is of interest that the ligation of K α 1T to its specific Abs can result in the upregulation of fibrotic growth factors and lipid rafts are involved in this process [17]. Similar to VEGF, we also demonstrated 0.5 μ g/mL of K α 1T Abs with crosslinking also induced the up-regulation of other growth factors such as HB-EGF (4.6 fold), bFGF (6.2 fold), and TGF- β (7.2 fold) (Figure 1B). Importantly, treatment with 2 μ g/mL of control Abs to keratin following crosslinking with appropriate secondary Ab, did not cause the enhancement of growth factor expression. These results demonstrate that Ab-mediated ligation of K α 1T in NHBE cells results in induction of several growth factors (VEGF, HB-EGF, bFGF and TGF- β) which are considered to be important in fibrogenesis.

3.2. HIF-1 α and MAPKinase over expression upon treatment of NHBE with K α 1T Abs

Several signaling molecules including mammalian target of rapamycin (mTOR) [26], RAC-alpha serine/threonine-protein kinase (Akt-1) [27] and hypoxia inducible factor (HIF-1 α) [28] have been suggested to play a significant role in the upregulation of pro-fibrogenic pathways. In our current study, culturing of NHBE cells with Abs to K α 1T followed by crosslinker and Western blot analysis of the cell lysates demonstrated an upregulation of HIF-1 α (Figure 2A). Furthermore, densitometric analysis also demonstrated 8.6 $\pm$ 1.2 fold increase (Figure 2B) in the expression of HIF-1 α . However, no significant upregulation of total or active phosphorylated mTOR and PI3K was noted. Culturing of NHBE cells with control keratin Abs did not cause upregulation of any of these signaling molecules.

It has been shown that HIF-1 α activation is mediated through MAP Kinase pathway [29, 30]. We analyzed the possibility that K α 1T Abs induced pro-fibrogenesis through activation of the MAP Kinase. Towards this we treated NHBE cells with K α 1T Abs following

crosslinking with the secondary Ab and nuclear extracts were analyzed. Phosphorylation of MAP Kinases was measured at 5, 10 and 15 min after addition of K α 1T Abs to NHBE cell cultures. Fold increase of phosphorylated form of the protein is represented as the ratio of the increase of fraction of phosphorylated to unphosphorylated form over the non-K α 1T Abs treated cell cultures. As shown in figure 2, treatment with K α 1T Abs after 15 min caused activation of various MAP Kinase components including, p-p38 (2.1 ± 0.6), p-ERK-1/2 (1.8 ± 0.7), p-c-Jun (4.3 ± 1.8). This demonstrates a specific activation of MAP Kinases by K α 1T Abs.

3.3. Congruent change of growth factor expression with modulation of HIF-1 α expression

To test the effect on fibrogenesis with HIF-1 α downregulation, YC-1 (3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole) a specific inhibitor of HIF-1 α was used [31-33]. As shown in figure 3A, pre-incubation of NHBE cells with YC-1 (100 μ M) for 6 hrs blocked the cross-linked K α 1T Abs mediated upregulation of HIF-1 α (1.3 ± 0.3 fold vs 8.6 ± 1.2 , by densitometric analysis). A dose titration curve of YC-1 from 0.1 to 100 μ M (Figure 3B) demonstrates a dose dependent decrease in the protein levels of HIF-1 α . However, there was no change in the phosphorylation ratio of p-p38, p-ERK-1/2 and p-c-JNK components of MAP Kinase complex analyzed in the nuclear extracts of NHBE cell cultures (Figure 3C). To further determine the effect of HIF-1 α downregulation on fibrogenesis, we analyzed the growth factor expression. As shown in figure 3D, down regulation of HIF-1 α with 100 μ M YC-1 resulted in down regulation of all pro-fibrogenic growth factors namely VEGF, HB-EGF, bFGF and TGF- β . These results strongly suggest that MAP Kinase pathway acts upstream of HIF-1 α during its induction.

To further determine the impact of HIF-1 α on the pro-fibrogenesis, we chemically inhibited the HIF-1 α degradation by blocking the enzyme PHD. Enzymatic activity of PHD has been shown to degrade HIF-1 α [34, 35]. DMOG is a cell permeable chemical molecule and is a competitive inhibitor of PHD and thus up regulates the intracellular concentration of HIF-1 α [36-38]. As shown in figure 4A, pre-treatment with DMOG (1 mM, for 6 hrs) and latter activation of NHBE cells with cross-linked K α 1T Abs caused a modest 11.8 ± 3.1 fold upregulation (from 8.6 ± 1.2 fold) of HIF-1 α . Titration with various concentrations from 0.001 mM to 1 mM of DMOG (Figure 4B) were performed and a final concentration of 0.1 and 1 mM was employed which demonstrated increase in HIF-1 α . There was a no significant change in activation of p-p38, p-ERK-1/2 and p-JNK in the presence of DMOG from the nuclear extracts of NHBE cell cultures (Figure 4C). To assess the fibrogenic effect of DMOG induced HIF-1 α upregulation, we also analyzed the growth factor levels in the DMOG treated NHBE cell cultures. As shown in figure 4D, treatment with DMOG of K α 1T Abs activated NHBE cells caused enhanced expression of VEGF (14.2 ± 3.1 fold vs 9.2 ± 2.1), HB-EGF (7.1 ± 1.9 fold vs 4.6 ± 1.2), bFGF (6.2 ± 1.8 fold vs 9.1 ± 2.3) and TGF- β (7.2 ± 2.3 fold vs 12.2 ± 3.1). These results demonstrate a direct pro-fibrogenic effect of HIF-1 α upregulation induced by ligation of K α 1T on AECs.

3.4. Inhibition of c-JNK of MAP Kinase causes significant down regulation of the growth factor expression profile

To investigate the role of JNK MAP Kinase pathway on pro-fibrogenesis we employed SP600125 (1,9-Pyrazoloanthrone) which is a well defined specific inhibitor of c-JNK/MAP Kinase [39, 40]. As shown in figure 5A, pre-incubation with SP600125 (50 μ M for 30 min) addition to K α 1T Abs treated NHBE cells prevented phosphorylation ratio of c-JNK without any effect on the other MAP kinases including p- p38 and p-ERK-1/2. Furthermore, inhibition of c-JNK caused significant ($p < 0.05$) downregulation of HIF-1 α (2.3 ± 1.2 fold vs 8.6 ± 1.2 ; Figure 5C) protein expression. Dose titration of SP600125 from 0.1-100 μ M showed a dose dependent inhibition of c-JNK phosphorylation ratio and also a decrease in

HIF-1 α (Figure 5B). Inhibition of c-JNK mediated HIF-1 α also caused significant attenuation of VEGF, HB-EGF, bFGF and TGF- β transcript levels (Figure 5D). Therefore, these data show that c-JNK MAP Kinase is likely acting upstream to promote HIF-1 α mediated fibrogenesis.

To further demonstrate the specificity of MAP Kinase pathway on pro-fibrogenesis we determined the transcription factors involved in the HIF-1 α induced upregulation of fibrogenesis. Towards this, we blocked the p38 transcriptional factor of MAP Kinase using SB 203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) which is a specific p38 targeting inhibitor of MAP Kinase [39, 40]. As shown in figure 4A, SB 203580 (10 μ M, for 30 min) and latter addition of K α 1T Abs to NHBE cells following crosslinking caused decreased phosphorylation ratio of p38. However, there was no effect on other MAP Kinases including p-ERK-1/2 and p-JNK. Furthermore, SB 203580 did not cause any down regulation of HIF-1 α expression (Figure 5C) and the pro-fibrogenic growth factors (Figure 5D) indicating that p38 is not directly involved in the HIF-1 α mediated activation of pro-fibrogenic cascade. To demonstrate the effect of ERK-1/2 MAP Kinase pathway on pro-fibrogenesis we used U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate) which is a specific ERK-1/2 targeting inhibitor of MAP Kinase [39, 40]. As shown in figure 5A, U0126 (10 μ M for 30 min) and latter addition of K α 1T Abs to NHBE cells caused complete inhibition of phosphorylation ratio of ERK-1/2 with no effect of other MAP kinases including p-p38 and p-JNK. Dose titration of SB 203580 and U0126 was performed and 10 μ M was determined to be optimal inhibition concentration for the respective nuclear factors (data not shown). Similar to p38 inactivation, specific ERK-1/2 inactivation also did not result in down regulation of HIF-1 α (Figure 5C) and the pro-fibrogenic growth factors (Figure 5D). This shows that p38 and ERK-1/2 may not play a significant role in HIF-1 α mediated upregulation of pro-fibrogenic growth factors.

4. Discussion

The airway epithelium plays a critical role in the defenses of the lung against pathogens and particulates inhaled from the environment [41]. Importantly, AECs are central to the pathogenesis of major lung diseases, including interstitial pulmonary fibrosis, chronic obstructive pulmonary disease, and cystic fibrosis [42]. Previous studies from our laboratory have shown that lung transplant recipients diagnosed with chronic rejection (bronchiolitis obliterans syndrome, BOS(+) patients) often develop Abs reactive to epithelial cell self antigen, K α 1T [16]. Furthermore, development of Abs to K α 1T following human lung transplantation precedes the onset of BOS [16] suggesting a pathogenic role for these Abs. Recently, we demonstrated that lipid raft mediated binding of the both mAbs against K α 1T and serum from BOS(+)/K α 1T Abs(+) patients to the AECs resulted in the increased expression of pro-fibrogenic growth factors which is central in the immunopathogenesis of BOS [17]. In our current study we demonstrate for the first time that surface ligation of K α 1T expressed on AECs leads to HIF1 α -mediated signaling cascade that results in increased expression of several important fibrogenic growth factors which are considered to be important in the pathogenesis of chronic rejection.

To demonstrate that specific ligation of K α 1T by its specific Abs induced pro-fibrogenic HIF-1 α signaling on NHBE cells, we chemically blocked HIF-1 α with YC-1. Upon specific chemical inhibition of HIF-1 α there was decrease in pro-fibrotic growth factor levels with no change in MAP Kinases. It is of interest to note that YC-1 was also used by others to inhibit renal fibrosis in murine model for hypoxic tubular epithelial mediated interstitial fibrosis [43]. YC-1 has also shown to downregulate HIF-1 α signaling induced VEGF mediated retinal angiogenesis [44]. However, inhibition of c-JNK caused significant

inhibition of both HIF-1 α and growth factor expression indicating that activation of c-JNK/MAP Kinase is an upstream prerequisite for HIF-1 α protein expression. These results strongly suggest a role for HIF-1 α signaling in the fibrinogenesis processes seen in lung transplant recipients suffering from chronic rejection.

Although the exact role of HIF-1 α in inflammation and chronic rejection are yet to be defined, HIF-1 α has been shown to play a crucial role in the pro-fibrogenesis leading to fibrotic events in liver [45], kidney [43], adipose tissue [46] and lung [14]. Peyssonnaud et al demonstrated a central role for HIF-1 α in mediating a pro-inflammatory TLR-4 (toll like receptor-4) response in promoting LPS-induced sepsis in animal models [47]. In contrast, Ben-Shoshan et al using siRNA techniques have suggested a potential HIF-1 α induced Treg stimulation under hypoxic conditions for the anti inflammatory action [48]. Furthermore, using animal models of lung transplantation, Jiang et al have shown that in adenovirus mediated gene transfer of HIF-1 α through its pro-angiogenic role promotes allograft survival [15]. These apparent contradictory findings illustrates that the HIF-1 α induced inflammation is dependent on the micro environment and cytokine promoters leading to its further stimulation. We attribute this apparently opposing evidence to the unique dual role of HIF-1 α in being both pro-angiogenic and pro-fibrotic. We propose that HIF-1 α signaling in the initial period following transplantation promotes allograft survival due to its pro-angiogenic property, while hypoxia and oxidative stress during latter stages following transplantation resulting in thickening of airway epithelium as seen in BOS may be due to self-perpetuating positive feed-back towards upregulation of HIF-1 α leading to activation of fibrogenic cascade. However, given the *in situ* nature of our current work and the need for the linker molecule to promote the formation of gap junctions in the cell cultures, more studies using knock-out animal models are warranted to identify the causal-effect of HIF-1 α in chronic rejection following human lung transplantation. Furthermore, the reported dual role of this molecule towards both pro-angiogenesis mediating enhanced graft survival and pro-fibrosis resulting in chronic graft rejection warrants further studies to identify the mechanism of action by this molecule with respect to the development of chronic rejection.

In conclusion, we demonstrate that upon surface ligation of K α 1T present on epithelial cells with its specific Abs result in the activation of c-JNK/MAP Kinase. This activation of c-JNK/MAP Kinase causes increased downstream protein expression of HIF-1 α . This HIF-1 α signaling cascade leads to enhanced expression of several important pro-fibrogenic growth factors including VEGF, HB-EGF, bFGF and TGF- β , leading candidates for the pathogenesis of fibrotic events, which is the hallmark lesion of chronic rejection including BOS. Although our current studies are limited to *in vitro* analysis of normal human bronchial epithelial cell line, we propose that our findings will have a broader impact for the central role of HIF-1 α signaling on the fibrotic injury following other solid organ transplants as well as various respiratory diseases in which fibrosis and narrowing of lumen results in the pathology. It is significant that HIF-1 α inhibition is currently under investigation as a potential anti-cancer target [31, 49] and therefore can be readily translated into pulmonary diseases including for the treatment of chronic rejection following human lung transplantation.

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Highlights

- Defines the role of Abs to K α 1T in patients with BOS following human lung transplantation.
- Demonstrate that K α 1T Abs signal through HIF-1 α pathway leading to pro-fibrotic cascade.
- A direct role for immune response to a self-antigen plays a crucial role in chronic rejection.

Figure 1.

Activation of NHBE cells with K α 1T Abs causes upregulation of growth factor expression. (A) Dose dependent changes in the mRNA expression of VEGF was analyzed by RT-PCR with culturing NHBE cells in varying concentration (0.1 to 100 $\mu\text{g/mL}$) of mouse mAbs IgG K α 1T + 0.5 $\mu\text{g/mL}$ anti-mouse IgG (1); mouse mAbs IgG K α 1T (2); 0.1 to 100 $\mu\text{g/mL}$ mouse mAbs IgG keratin coincubated with 0.5 $\mu\text{g/mL}$ anti-mouse IgG (3); and anti-mouse IgG (4). (B) NHBE cells cultured with 0.5 $\mu\text{g/mL}$ of mouse mAbs IgG K α 1T for 48 hrs. mRNA expression profile of VEGF, HB-EGF, bFGF and TGF- β was analyzed by RT-PCR; Parallel experiments performed with NHBE cultures with 0.5 $\mu\text{g/mL}$ of mouse mAbs IgG K α 1T coincubated with 0.5 $\mu\text{g/mL}$ anti-mouse IgG for 48 hrs. Control experiments were performed with only 0.5 $\mu\text{g/mL}$ of mouse mAbs IgG K α 1T, 0.5 $\mu\text{g/mL}$ anti-mouse IgG; and 2 $\mu\text{g/mL}$ mouse mAbs IgG keratin coincubated with 5 $\mu\text{g/mL}$ anti-mouse IgG. Data are mean values $\pm$ SEM from four independent experiments.

Figure 2.

Effect of the signal molecules (HIF-1 α , mTOR, PI3K and MAP Kinases) upon activation of NHBE cells with K α 1T Abs. NHBE cells were cultured individually with 0.5 μ g/mL of mouse mAbs IgG K α 1T + 0.5 μ g/mL anti-mouse IgG; 0.5 μ g/mL anti-mouse IgG; and 2 μ g/mL mouse mAbs IgG keratin + 5 μ g/mL anti-mouse IgG. (A) Western blot analysis was performed to determine the protein level expression of HIF-1 α , mTOR and Akt-1. mTOR and Akt-1 were analyzed for both total and active phosphorylated forms 15 min after K α 1T Abs treatment (B) Densitometric analysis to determine quantitative expression of HIF-1 α , mTOR and PI3K. (C) Western blot analysis was performed to determine the protein level expression of p38, ERK-1/2, and c-JNK for both total and active phosphorylated forms after 15 min stimulation; (D) Densitometric analysis to determine quantitative expression of p38, ERK-1/2, c-JNK, and AP-1; (E) The expression of p-c-JNK with 0.5 μ g/mL of mouse mAbs IgG K α 1T + 0.5 μ g/mL anti-mouse IgG at 5,10, 15 and 30 min. 'Fold increase' on densitometric analysis is defined in Materials and Methods under statistical analysis section. (*) p <0.05; Data are mean values $\pm$ SEM from four independent experiments.

Figure 3.

Effect of modulation of HIF-1α by decreasing the expression HIF-1α by specific inhibitor, YC-1 (3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole) on Kα1T Abs mediated activation of NHBE cells. (A) The NHBE cells were cultured in the presence of 100μM of YC-1 for 6hrs followed by addition of 0.5μg/mL of mouse mAbs IgG Kα1T + 0.5μg/mL anti-mouse IgG. The control groups include NHBE cells cultured in the presence of 100μM of YC-1 for 6hrs followed by addition of either 0.5μg/mL of mouse mAbs IgG Kα1T only, or 0.5μg/mL anti-mouse IgG only, or absence of both. Western blot analysis was performed to determine the protein level expression of HIF-1α, p38, ERK-1/2, and c-JNK. p38, ERK-1/2, and c-JNK were probed for both total (data not shown) and active phosphorylated forms; (B) Dose-titration of YC-1 on the HIF-1α expression; (C) Densitometric analysis to determine quantitative expression profile of HIF-1α, p38, ERK-1/2, and c-JNK; and (D) mRNA expression profile of VEGF, HBEGF, bFGF and TGF-β. mRNA expression reported fold increase of individual molecule in the presence of 0.5 μg/mL of mouse mAbs IgG Kα1T + 0.5μg/mL anti-mouse IgG over the absence of Abs+ 100 μM of YC-1 over absence of Abs after 48 hrs. 'Fold increase' on densitometric analysis is defined in 'Materials and Methods' section. (*) p <0.05; Data are mean values ± SEM from four independent experiments.

Figure 4.

Effect of modulation of HIF-1 α expression by increasing the expression of HIF-1 α by inhibition of PHD, known to breakdown HIF-1 α with PHD inhibitor, DMOG on K α 1T Abs activated of NHBE cells. (A) The NHBE cells were cultured in the presence of 1 mM DMOG for 6hrs followed by addition of 0.5 μ g/mL of mouse mAbs IgG K α 1T + 0.5 μ g/mL anti-mouse IgG. The control groups include NHBE cells cultured in the presence of 1 mM DMOG for 6hrs followed by addition of either 0.5 μ g/mL of mouse mAbs IgG K α 1T only, or 0.5 μ g/mL anti-mouse IgG only, or absence of both. Western blot analysis was performed to determine the protein level expression of HIF-1 α , p38, ERK-1/2, and c-JNK. p38, ERK-1/2, and c-JNK were probed for both total (data not shown) and active phosphorylated forms; (B) Dose-titration of PHD inhibitor (DMOG) on the HIF-1 α expression; (C) Densitometric analysis to determine quantitative expression profile of HIF-1 α , p38, ERK-1/2, and c-JNK; and (D) mRNA expression profile of VEGF, HB-EGF, bFGF and TGF- β . mRNA expression reported fold increase of individual molecule in the presence of 0.5 μ g/mL of mouse mAbs IgG K α 1T + 0.5 μ g/mL anti-mouse IgG over the absence of Abs+ 1mM of DMOG (PHD inhibitor) over absence of Abs after 48 hrs. 'Fold increase' on densitometric analysis is defined in Materials and Methods under section. (*) $p < 0.05$; Data are mean values $\pm$ SEM from four independent experiments.

Figure 5.

Effect of MAP Kinase on K α 1T Abs mediated activation of NHBE cells. (A) NHBE cells were cultured individually with 50μM of c-JNK inhibitor (SP600125, 1,9-Pyrazoloanthrone) to 30min; 0.5μg/mL of mouse mAbs IgG K α 1T + 0.5μg/mL anti-mouse IgG for 15 min and preincubated with 50μM of c-JNK inhibitor for 30min; and 0.5μg/mL of mouse mAbs IgG K α 1T + 0.5μg/mL anti-mouse IgG for 15 min and preincubated with 50μM of c-JNK inhibitor to 30 min. Western blot analysis was performed to determine the protein level expression of HIF-1α, p38, ERK-1/2, and c-JNK. p38, ERK-1/2, and c-JNK were probed for both total (data not shown) and active phosphorylated forms; Similarly for p38 and ERK-1/2, 10μM of p38 inhibitor (SB 203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) to 30min and 10μM of ERK-1/2 inhibitor (U0126, 1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene monoethanolate) to 30min were used. (B) Effect of dose titration of c-JNK inhibitor and effect of HIF-1α expression, and c-JNK phosphorylation. (C) Densitometric analysis to determine quantitative expression profile of HIF-1α, p38, ERK-1/2, and c-JNK; (D) mRNA expression profile of VEGF, HB-EGF, bFGF and TGF-β. mRNA expression reported fold increase of individual molecule in the presence of 0.5μg/mL of mouse mAbs IgG K α 1T + 0.5μg/mL anti-mouse IgG over the absence of Abs+ 10μM of either c-JNK inhibitor over absence of Abs after 48 hrs stimulation. For C and D, dark grey represents no inhibitor addition, crossed line fill represents c-JNKinhibition, and light grey represents p38 inhibition and straight line fill represents ERK-1/2 inhibition. 'Fold increase' on densitometric analysis is defined in Materials and Methods under statistical analysis section. (*) p < 0.05; Data are mean values ± SEM from four independent experiments.