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Lipid raft facilitated ligation of K-α1-tubulin by specific antibodies on epithelial cells: Role in pathogenesis of chronic rejection following human lung transplantation

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ABSTRACT

Long term function of human lung allografts is hindered by development of chronic rejection manifested as Bronchiolitis Obliterans Syndrome (BOS). We have previously identified the development of antibodies (Abs) following lung transplantation to K-α1-tubulin (KAT), an epithelial surface gap junction cytoskeletal protein, in patients who develop BOS. However, the biochemical and molecular basis of the interactions and signaling cascades mediated by KAT Abs are yet to be defined. In this report, we investigated the biophysical basis of the epithelial cell membrane surface interaction between KAT and its specific Abs. Towards this, we analyzed the role of the lipid raft-domains in the membrane interactions which lead to cell signaling and ultimately increased growth factor expression. Normal human bronchial epithelial (NHBE) cells, upon specific ligation with Abs to KAT obtained either from the serum of BOS(+) patients or monoclonal KAT Abs, resulted in upregulation of growth factors VEGF, PDGF, and bFGF (6.4 ± 1.1-, 3.2 ± 0.9-, and 3.4 ± 1.1-fold increase, respectively) all of which are important in the pathogenesis of BOS. To define the role for lipid raft in augmenting surface interactions, we analyzed the changes in the growth factor expression pattern upon depletion and enrichment with lipid raft following the ligation of the epithelial cell membranes with Abs specific for KAT. NHBE cells cultured in the presence of β-methyl cyclodextran (βMCD) had significantly reduced growth factor expression (1.3 ± 0.3, vs βMCD untreated being 6.4 ± 1.1-fold increase) upon stimulation with KAT Abs. Depletion of cholesterol on NHBE cells upon treatment with βMCD also resulted in decreased partitioning of caveolin in the membrane fraction indicating a decrease in raft-domains. In conclusion, our results demonstrate an important role for lipid raft-mediated ligation of Abs to KAT on the epithelial cell membrane, which results in the upregulation of growth factor cascades involved in the pathogenesis of BOS following human lung transplantation.

1. Introduction

Lung transplantation is considered the definitive treatment for patients with end-stage lung diseases [1]. The long term survival of the transplanted lung allograft is limited by chronic rejection clinically diagnosed as Bronchiolitis Obliterans Syndrome (BOS). Chronic rejection is a fibroproliferative process that involves inflammation and fibrosis of the lamina propria and lumen resulting in progressive decline in pulmonary function and eventual allograft failure. The incidence of BOS is approximately 50% within 3 years of transplantation [2]. Furthermore, the median survival after the diagnosis of BOS is only 3 years. Current immunosuppressive regimens can only slow the progression of BOS and can't reverse the pathology [3].

Although the pathogenesis of BOS is not yet fully defined, several risk factors have been associated with the development of chronic rejection, including recurrent/refractory episodes of acute rejection, cytomegalovirus (CMV) and other respiratory viral infections, human leukocyte antigen (HLA) mismatches, primary graft dysfunction, etc. In addition, several non-specific risk factors such as donor and recipient age, graft ischemic time, and bacterial/fungal/non-CMV viral infection have also been associated with decreased long term survival of the graft [4]. The development of Abs to donor HLA (Abs) has also been linked to lymphocytic bronchiolitis underscoring a potential role of humoral immunity in the development of chronic rejection [5]. Recent studies from our laboratory and others have also proposed a role for auto-Abs to self-antigens Collagen V (ColV) and K-α1-tubulin (KAT) in the pathogenesis of BOS following lung transplantation [6,7]

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suggesting a cross-talk mechanism between alloimmune and autoimmune responses in the immunopathogenesis of BOS [8].

Studies have shown that the airway epithelial cells (AECs) are the main target for the immunologic insult during the pathogenesis of allograft rejection [9]. Activation of epithelial cells by Abs to HLA can result in the production of growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and endothelin (ET)-1 [10,11]. Exposure to these growth factors can lead to the activation and proliferation of fibroblasts and smooth muscle cells. More significantly, *in vivo* studies have revealed a temporal relationship between elevated levels of growth factors and significant fibroblast migration and proliferation within the small airways [12]. Although, previous studies from our laboratory and others have implicated that the development of Abs to donor HLA predisposes patients to the development of chronic rejection, there are many incidences of lung transplant recipients with BOS where Abs to mismatched donor HLA can't be readily demonstrated, thus suggesting a role for Abs to non-HLA antigens in the pathogenesis of BOS [13,14].

We have recently demonstrated that Abs to KAT expressed on the epithelial cell surface play an important role in the pathogenesis of human lung transplant recipients diagnosed with BOS [7]. However, the molecular basis of the interaction of Abs to KAT and AECs and the mechanisms by which KAT Abs mediate fibroproliferation remain ill defined. In this report, we demonstrate that lipid rafts present on the surface of the epithelial cells are the critical first step leading to enhanced growth factor cascade, which is essential in the pathogenesis of BOS.

2. Materials and methods

2.1. Cell cultures

Normal human bronchial epithelial (NHBE) cells were obtained from the American Type Culture Collection (CRL-2503, ATCC, Manassas, VA) and cultured in small airway cell basal medium (CC-3119, Lonza, USA) supplemented by SAGM™ provided by the company (CC-4124, Lonza, USA). Cell lines were frozen at 70 °C until use. Upon thawing, cells were maintained in sterile 5% CO_2 incubator in the growth media at 37 °C.

2.2. Detection of KAT Abs by ELISA

The patients sera were tested for the development of Abs to KAT by enzyme linked immunosorbent assay (ELISA) developed in our laboratory. Recombinant human KAT (NM_006082) was purified from *Escherichia coli* expression vector stock. *E. coli* was cultured overnight at 37 °C with kanamycin and IPG (each at concentration of 1 ng/mL). The bacteria were centrifuged, lysed and protein purified on Ni-NTA column following manufacturer's instructions (PrepEase kits, Affymetrix/USB Corporation, OH). To perform ELISA, 96-well plates (Nunc, NY) were coated with 1 μg/mL purified KAT in phosphate-buffer solution (PBS) and incubated overnight at 4 °C. The antigen coated wells were blocked with 1% bovine serum albumin for 2 h. Sera were tested at dilutions of 1:500 for presence of Abs against KAT. Commercially available anti-KAT Abs (Santacruz Biotechnology, CA) were used as positive controls. For detection of specific binding, anti-human IgG, IgM bound to horseradish peroxidase (Jackson ImmunoResearch Laboratory, PA) was utilized and developed with tetramethylbenzidine substrate (Millipore, CA). Immunoabsorbance was detected at 460 nm and concentration of Abs was calculated based on a standard curve using the binding of known concentration of commercial anti-KAT Abs. The titers of Abs to self-antigens was determined using values obtained from calculating two standard deviations from the mean concentration of KAT Abs in healthy control subjects.

2.3. Modulation of cholesterol level on epithelial cells

The NHBE cells were enriched with or depleted of cholesterol by incubating them with methyl-β-cyclodextrin (βMCD) saturated with cholesterol or with βMCD alone (non-complexed with cholesterol) respectively [15]. Briefly, the cholesterol stock solution in chloroform:methanol (1:1, vol/vol) was added to a glass tube and the solvent was evaporated. Then, 5 mM βMCD solution in RPMI medium without serum was added to the dried cholesterol. The tube was vortexed, sonicated, and incubated overnight in a shaking bath at 37 °C. βMCD was saturated with cholesterol at a βMCD/cholesterol molar ratio of 8:1. In preparation for an experiment, cells were washed three times with serum-free RPMI medium. Cells were then incubated with cholesterol-saturated βMCD solution or with βMCD solution containing no cholesterol, or with a mixture of these for 120 min. During the incubation, cells were maintained in a humidified CO_2 incubator at 37 °C. After exposure to βMCD, cells were washed three times with serum-free media and incubated in serum-free media for at least 24 h, providing the time window for the electrophysiological recordings. To attain the intermediate cellular levels of cholesterol, cells were exposed to various mixtures of 5 mM βMCD saturated with cholesterol and 5 mM βMCD. βMCD and cholesterol were purchased from Sigma Chemical (St. Louis, MO).

2.4. Growth factor assay

Expression profiles of intracellular signal genes in the isolated NHBE were analyzed using the FAm-labeled RT-PCR primers for VEGF, PDGF, bFGF (Applied Biosystems, Foster City, CA) as per the manufacturer's recommendation. Briefly, total RNA was extracted from 10^6 cells using TRIzol reagent (Sigma–Aldrich). The RNA was reverse-transcribed and real-time PCR was performed in a final reaction volume of 20 μL using iCycler 480 Probes Master (Roche Diagnostics). Each sample was analyzed in triplicate. Cycling conditions consisted of an initial denaturation of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30s, followed by 61 °C for 1 min.

2.5. Western blot

Protein level of caveolin and α-tubulin were analyzed using the Western blot. The retrieved cells from the matrigels on day 30 were lysed using 4% SDS cell lysis buffer supplemented with protease inhibitor cocktail and EDTA. The lysates were boiled for 20 min in sample buffer (200 mmol/L Tris (pH6.8), 20% glycerol, 2% SDS, 0.1% bromophenol blue, and 10% β-mercaptoethanol and centrifuged for 30 min and run on 4–12% gradient Bis-Tris denaturing gel (Nupage, Invitrogen). The gel was transferred onto nitrocellulose membrane, and blocked overnight with 5% non-fat milk in PBS-T (0.1% Tween 20). Thereafter, the membrane was incubated for 1 h at room temperature with the appropriate Abs. After labeling with HRP-labeled secondary Abs (1:10,000 dilution, R&D systems), the membrane was developed using the chemiluminescence kit (Millipore) and analyzed on using Bio-Rad Universal Hood II (Hercules, CA). Morphometric analysis was done using the software provided by the company.

2.6. Statistical analysis

Data are expressed as mean ± SE. Statistical differences between means were analyzed using a paired or unpaired Student's *t* test. A value of *P* less than 0.05 was considered significant. All data