JNJ0966 inhibits PDGF-BB-induced airway smooth muscle cell proliferation and extracellular matrix production by regulating MMP-9

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Abstract
The increased proliferation and extracellular matrix (ECM) production of airway smooth muscle cells (ASMCs) are crucial factors in asthma progression. JNJ0966, one of the metalloproteinase-9 (MMP-9)-specific inhibitors, has been demonstrated to be involved in the progression and development of diversified diseases. Nevertheless, the function of JNJ0966 in ASMCs remains unclear. This study aimed at investigating the effects of JNJ0966 on asthma progression. In our study, the platelet-derived growth factor BB (PDGF-BB) was first utilized to stimulate the cell model for asthma. Results demonstrated that the cell viability of ASMCs was increased by PDGF-BB (0, 10, 20, and 30 ng/mL) in a dose-dependent manner. Further investigation revealed that JNJ0966 inhibited the cell activity and migration ability of PDGF-BB-induced ASMCs. In addition, JNJ0966 relieved ECM deposition in PDGF-BB-induced ASMCs. Finally, through rescue assays, the results showed that overexpression of MMP-9 reversed the inhibitory effects of JNJ0966 on cell viability and ECM deposition in ASMCs. In conclusion, our findings suggested that JNJ0966 inhibited PDGF-BB-induced ASMC proliferation and ECM production by modulating MMP-9. These findings might provide novel insight for the treatment of asthma.

KEYWORDS
ASMCs; asthma; JNJ0966; MMP-9; PDGF-BB

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Introduction

Bronchial asthma (asthma for short) is a common noninfectious chronic disease that seriously endangers human health.1,2 Around 334 million people worldwide suffer from asthma, with more than 45 million in China.3 It is expressed by panting, coughing, breathing hard, and chest tightness.4 If these symptoms cannot be ameliorated promptly, asthma patients may die.4,5 In asthma, the abnormal proliferation of ASMCs can result in tracheal remodeling.6,8 At present, there are few effective airway remodeling therapy methods for the treatment of asthma. Thus, exploring the molecular mechanism of abnormal proliferation and ECM production of ASMCs will contribute to figuring out this predicament.

Airway remodeling is a hinge feature of continuous asthma and involves an increase in airway smooth muscle (ASM) volume.9,10 This increase is partly due to the increased protein deposition of ECM surrounding ASM and the increased hyperplasia of ASM.11,12 After activation of cytokines (such as PDGF, VEGF, EGF, and TGF-β), ASM can become hypertrophic, accompanied by enhanced cell migration.13 Moreover, cytokines can act as the corresponding receptors of ASMCs to activate intracellular pathways such as MAPK/ERK, JNK, P38, and PI3K/AKT, thereby inducing cell mitosis and increasing the proteins that regulate cell migration, such as MMP-9, NOR1, etc.14,16 The proliferative and hypertrophic smooth muscle not only thickens the airway wall, causes airway stenosis, and reduces the contractility of the airway wall but also increases the synthesis of ECM proteins and exacerbates the fibrosis of the airway wall.17

MMP-9 is a matrix metalloproteinase that plays a vital role in tumor metastasis.18 Studies demonstrated that the expression of MMP-9 in plasma of asthmatic patients was observably higher than that of the controls.19,20 JNJ0966 is a novel inhibitor of MMP-9 that highly selectively inhibits proMMP-9 activation into its active form MMP-9 by interacting with the structural pocket of the MMP-9 proenzyme cleavage site near Arg106.21,22 A previous study showed that JNJ0966 has therapeutic effects on some diseases, and it can effectively reduce the severity of autoimmune encephalomyelitis in mice models.21 In gastric cancer cells, JNJ0966 downregulates MMP-9 to decrease cell proliferation and migration.23 However, the effects of JNJ0966 on ASMCs have not been elucidated.

In this study, we found that JNJ0966 can suppress the cell activity and migration ability of ASMCs induced by PDGF-BB and reduce ECM deposition. Moreover, over-expression of MMP-9 can reverse the inhibitory effects of JNJ0966 on asthma progression.

Methods

Cell culture

The human airway smooth muscle cells (ASMCs) were acquired from the Sciencell Research Laboratories (cat. no. 3400; Carlsbad, CA, USA). The ASMCs were cultured in the Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) with 20% fetal bovine serum (FBS, Gibco, USA) in an incubator at 37°C with 5% CO₂. PDGF-BB (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) with different concentrations (0, 10, 20, and 30 ng/mL) was applied for the treatment of ASMCs for 24 h. JNJ0966 (0, 5, 10, and 20 μM) purchased from ChemBridge Corporation was applied for the treatment of ASMCs.

Cell transfection

The full length of MMP-9 was subcloned into the pcDNA3.1 vectors (Sangon Biotech, China), and the empty pcDNA3.1 vector was utilized as the control. Cells were transfected with these above vectors through Lipofectamine 2000 (Invitrogen, USA). Then, 48 h after transfection, cells were collected for further investigation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

ASMCs (1×10⁴ cells/well) were added to 96-well plates for culture. After 48 h, 10 μL of MTT (Invitrogen) was added to each well. Formazan was dissolved with DMSO (100 μL; Sigma, USA). The absorbance at 490 nm was detected on a microplate reader.

Wound-healing assay

The ASMCs were plated into six-well plates until a 80%-90% confluence was reached. Next, the scratch was done with a sterile 100-μL pipette tip. Cells were then grown for another 24 h. Microscopy images were captured through an inverted microscope (Leica, Wetzlar, Germany) at 0 and 24 h.

RT-qPCR

Total RNA was extracted from ASMCs with the TRIzol reagent (Sigma, USA). The cDNAs were generated using the PrimeScript™ RT reagent kits (Takara, Dalian, China). GAPDH was used as a reference. RT-qPCR was conducted using the SYBR Prime Script RT-PCR kit (TaKaRa, China). The primers were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>MMP-9</td>
<td>5′-TGGTGCTTCTTGCTTTGGTATCGTGGAAGG-3′</td>
<td>5′-GCCACTCACTGAGTCAAAACGAG-3′</td>
</tr>
<tr>
<td>NOR1</td>
<td>5′-GTTTGTTACACACTGAGCCGAAATCC-3′</td>
<td>5′-ATCGGACGGTATGAGAAGAAT-3′</td>
</tr>
<tr>
<td>COL I</td>
<td>5′-CTTCTTGTTACACACTGAGCCGAAATCC-3′</td>
<td>5′-ATCGGACGGTATGAGAAGAAT-3′</td>
</tr>
<tr>
<td>COL III</td>
<td>5′-CTTCTTGTTACACACTGAGCCGAAATCC-3′</td>
<td>5′-ATCGGACGGTATGAGAAGAAT-3′</td>
</tr>
<tr>
<td>FIBRONECTIN</td>
<td>5′-AAGACCCAGCAGCAGCAAGTCGGG-3′</td>
<td>5′-AAGACCCAGCAGCAGCAAGTCGGG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-ACAAA CTTTTGTTATGTTGGAAGAGG-3′</td>
<td>5′-GCCATACGCGAGAGGAG-3′</td>
</tr>
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</table>

The primers were designed using the Primer3 software. Amplification was carried out using the SYBR Green PCR kit (TaKaRa, China) on a CFX96 real-time PCR system (Bio-Rad, Hercules, USA). The expression of each gene was normalized to GAPDH expression. The fold change was calculated according to the 2⁻ΔΔCt method.

Western blot

The ASMCs were lysed using the RIPA buffer (Thermo Scientific, Waltham, USA). The isolated proteins were transferred to poly(vinylidene fluoride) membranes after being separated by SDS-PAGE. The membranes were incubated with primary antibodies, such as MMP-9 (1:1000, ab38898; Abcam), collagen I (1:1000, ab34710; Abcam), collagen III (1:5000, ab7778; Abcam), fibronectin (1:1000, ab2413, Abcam), and GAPDH (1:1000, ab9485; Abcam) at 4°C overnight. Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Finally, the protein bands were evaluated using the ECL kit (Thermo Scientific, Waltham, USA). The protein bands were analyzed using the ImageJ software.
Waltham, MA, USA). Then, images were analyzed using ImageJ software. With GAPDH being used as an internal reference, the relative expression level of the protein was the gray value of the band /the gray value of the GAPDH band.

Statistical analysis

The analysis of data was done using SPSS 21.0 software (IBM, Armonk, NY, USA). The experiments were repeated three times, and the data were expressed as mean ± SD (standard deviation). The normality and homogeneity of data were, respectively, analyzed through the Shapiro–Wilk and Levene’s tests. The differences among groups were compared through the Student’s t test (two) or one-way analysis of variance (ANOVA) (more than two). The value of $p < 0.05$ was regarded as statistically significant.

Results

JNJ0966 weakened the cell activity of PDGF-BB-induced ASMCs

JNJ0966 has been demonstrated to repress tumorigenesis. However, its role in the cell viability and ECM deposition in ASMCs remains unclear. As displayed in Figure 1A, the cell viability was increased by PDGF-BB (0, 10, 20, and 30 ng/mL) treatment in a dose-dependent manner. Moreover, the increased cell viability of ASMCs mediated by PDGF-BB (20 ng/mL) was inhibited by treating with JNJ0966 (Figure 1B). Taken together, JNJ0966 weakened the cell activity of PDGF-BB-induced ASMCs.

JNJ0966 reduced the migration ability of PDGF-BB-induced ASMCs

In addition, the relative wound width was reduced by the PDGF-BB treatment, but this change was reversed by JNJ0966 treatment (Figure 2), suggesting that the enhanced migration ability of PDGF-BB-induced ASMCs was reduced by JNJ0966 treatment.

Figure 1  JNJ0966 weakened the cell activity of PDGF-BB-induced ASMCs. (A) The cell viability was examined through MTT assay in ASMCs induced by different concentrations of PDGF-BB (0, 10, 20 and 30 ng/mL). (B) The cell viability was detected through MTT assay in the Control, PDGF-BB, PDGF-BB+JNJ0966 (5 μM), PDGF-BB+JNJ0966 (10 μM), and PDGF-BB+JNJ0966 (20 μM) groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 2  JNJ0966 reduced the migration ability of PDGF-BB-induced ASMCs. The cell migration ability was assessed through wound healing assay in the Control, PDGF-BB, PDGF-BB+JNJ0966 (5 μM), PDGF-BB+JNJ0966 (10 μM), and PDGF-BB+JNJ0966 (20 μM) groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$. 
JNJ0966 relieved the ECM deposition in PDGF-BB-induced ASMCs

Further investigation showed that the mRNA expressions of collagen I, collagen III, and fibronectin were upregulated, but this effect was attenuated by treatment with JNJ0966 (Figure 3A). Similarly, the increased protein expressions of collagen I, collagen III, and fibronectin triggered by PDGF-BB (20 ng/mL) were reversed by JNJ0966 treatment (Figure 3B,C). Moreover, the enhanced levels of TNF-α, IL-1β, and IL-6 were attenuated by JNJ0966 treatment (Figure 3D). These above data revealed that JNJ0966 relieved the ECM deposition in PDGF-BB-induced ASMCs.

Overexpression of MMP-9 reversed the inhibitory effects of JNJ0966 on cell viability and ECM deposition in ASMCs

The overexpression efficiency of pc-MMP-9 was confirmed in Figure 4A. Next, the rescue assays were done to investigate the function of JNJ0966 in ASMCs. The cell viability was enhanced after overexpressing MMP-9, and the weakened cell viability mediated by JNJ0966 could be rescued by overexpressing MMP-9 (Figure 4B). Moreover, the mRNA and protein expressions of collagen I, collagen III, and fibronectin were both upregulated by MMP-9 overexpression. The reduced mRNA and protein expressions of collagen I, collagen III, and fibronectin by JNJ0966 were offset after MMP-9 overexpression (Figure 4C-E). In summary, overexpression of MMP-9 reversed the inhibitory effects of JNJ0966 on cell viability and ECM deposition in ASMCs.

Discussion

Our findings revealed that JNJ0966 weakened the cell activity, migration ability, and ECM deposition of PDGF-BB-induced ASMCs. Furthermore, through rescue assays, it was found that overexpression of MMP-9 reversed the inhibitory effects of JNJ0966 on cell viability and ECM deposition in PDGF-BB-induced ASMCs.
Figure 4 Overexpression of MMP-9 reversed the inhibition of JNJ0966 on cell viability and ECM deposition in ASMCs. (A) Protein expression of MMP-9 was assessed through western blot after overexpressing MMP-9. (B) The cell viability was measured through MTT assay. (C) The mRNA expressions of collagen I, collagen III and fibronectin were measured through RT-qPCR. (D,E) Protein expressions of collagen I, collagen III, and fibronectin were detected through western blot. Groups were divided into the Control, PDGF-BB, PDGF-BB+JNJ0966, PDGF-BB+NC, PDGF-BB+pc-MMP-9, PDGF-BB+JNJ0966+NC, or PDGF-BB+JNJ0966+pc-MMP-9 group. PDGF-BB: 20 ng/mL; JNJ0966: 10 μM. *p<0.05, **p<0.01, ***p<0.001.
The airway remodeling causes changes in the airway structure, which leads to immune system confusion and asthma. Unfortunately, airway remodeling is considered to be causally related to asthma, but there is no better way to treat asthma besides airway remodeling. It is confirmed that airway remodeling caused by ASMc proliferation can exacerbate the severity of asthma. Therefore, restraining cell proliferation may attenuate airway remodeling.

Platelet-derived growth factor BB (PDGF-BB) is a dimer subtype excreted by inflammatory cells and airway epithelial cells. PDGF-BB has been noted as the fully upregulated inflammatory mediator in asthma. PDGF-BB upregulation results from ASMc hyperplasia. In our study, PDGF-BB was first utilized to stimulate a cell model for asthma. Results demonstrated that the cell viability of ASMcs was enhanced by PDGF-BB (0, 10, 20, and 30 ng/mL) in a dose-dependent manner. JNJ0966 is a highly selective compound, which specifically inhibits the activation of MMP-9 zymogen and the production of catalytically active enzymes. In this work, further investigation revealed that JNJ0966 weakened the cell activity and migration ability of PDGF-BB-induced ASMcs.

Extracellular matrix (ECM) makes a multifaceted system for the mutual effects among a variety of glycoproteins, proteoglycans, stromal cell molecules, structural proteins, and various hyaluronic acids, and these factors cooperate and combine to generate bioactive polymers. It is well known that proliferation and ECM generation of ASMcs play vital roles in the evolution of airway remodeling. For example, miR-204-5p targets Six1 to suppress TGFB-β1-mediated proliferation and ECM production of ASMcs in asthma. MiR-143-3p modulates NFATc1 expression to affect TGFB-β1-triggered cell proliferation and ECM deposition in ASMcs. AMOTL2 reduces YAP1 expression to repress TGFB-β1-mediated proliferation and ECM generation in ASMcs. Eosinophils facilitate ASMc proliferation and ECM production through upregulating WNT-5a and TGFB-1 in asthma. In this study, we found that JNJ0966 relieved the ECM deposition in PDGF-BB-induced ASMcs.

MMP-9 also has been affirmed to exhibit a pivotal function in various diseases. For instance, G6PD contributes to clear cell renal cell carcinoma tumorigenesis by enhancing MMP9. Additionally, enhanced MMP9 expression facilitates diabetic osteoarthritis development. Moreover, IncRNA UCA1 epigenetic regulates MMP9 to affect ASMc proliferation and migration. ORMDL3 modulates the ERK/MMP-9 pathway in asthma to affect airway remodeling. Importantly, the expression of MMP-9 in airways has been identified as a monitor marker for asthma. In our study, through rescue assays, it was found that overexpression of MMP-9 reversed the inhibitory effects of JNJ0966 on cell viability and ECM deposition in ASMcs.

In summary, we first confirmed that JNJ0966 inhibited PDGF-BB-induced ASMc proliferation and ECM production by modulating MMP-9. However, our findings regarding the effects of JNJ0966 on asthma remain limited. In the future, more experiments will be carried out to further investigate JNJ0966 in asthma progression.

Competing Interests
The authors declare no conflict of interest to disclose.

Author Contributions
Yingying Zhang designed and carried out the experiments. Fan Yao analyzed and interpreted the data, and Zhihua Qin prepared the manuscript with contributions from all co-authors.

Reference
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