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Original Article

The C-jun N-terminal kinase signaling pathway regulates cyclin D1 and cell cycle progression in airway smooth muscle cell proliferation

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Abstract: Background: Airway smooth muscle cell (ASMC) proliferation is a central feature of asthmatic airways and is elicited by mechanisms of considerable interest. While the roles of the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways in ASMC proliferation are well established, the mechanisms by which the c-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and nuclear factor-kappa B (NF-κB) pathways contribute are still relatively obscure. Methods: Normal human ASMCs, asthmatic human ASMCs, and rat ASMCs were cultured in vitro. The effects of JNK, p38, and NF-κB inhibition on ASMC proliferation induced by fetal bovine serum (FBS) were estimated by crystal violet assay and tritiated thymidine uptake. The involvement of cell cycle regulators in ASMC proliferation was examined using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blotting. Cell apoptosis and cell cycle were analyzed by flow cytometry. Results: The inhibition of JNK by the chemical inhibitor SP600125 and by small interfering RNA (siRNA) strongly suppressed ASMC proliferation. The p38 MAPK inhibitors SB203580 and NF-κB sc-3060, meanwhile, had little effect. SP600125 significantly decreased the number of cells in the S and G2/M phases of the cell cycle, as well as cyclin D1 protein levels in human ASMCs. SP600125 elicited no changes in the rate of apoptosis. Conclusions: We conclude that the JNK pathway contributes to human ASMC proliferation via cyclin D1 levels and the regulation of the cell cycle.

Keywords: Airway remodeling, airway smooth muscle, asthma, cyclin D1, JNK

Introduction

Asthma is characterized by airflow limitation, airway hyperresponsiveness (AHR), and chronic airway inflammation. All of these conditions are associated with airway remodeling, a process that changes the structure of the asthmatic airways [1, 2]. The increased airway smooth muscle (ASM) mass is one of the most important determinants of the alterations in the structure of the airways [3]. An understanding of the mechanisms of the increase in ASM mass may therefore open doorways to the development of new treatments for preventing or reversing airway remodeling in asthma. Investigators have established that ASM cell (ASMC) hyperplasia [4] and ASMC hypertrophy [5] both initiate processes leading to increased ASM mass. The former, ASMC hyperplasia, results from increased rates of cell division [6] and/or decreased rates of apoptosis [7]. As for intracellular signaling, most reports concur that extracellular signal-regulated kinase (ERK) and phosphoinositide 3 kinase (PI3K) activation are the major signal transduction pathways for ASMC proliferation via the regulation of cyclin D1 expression [8-13].

ERK, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) belong to the mammalian family of MAPKs. MAPKs contribute to various cellular processes from the production of inflammatory cytokines to the regulation of proliferative pathways [14]. Most studies on the MAPK family in relation to ASMCs have focused on the role of the p38 MAPK- and ERK-mediated pathways [15]. Broadly speaking, the p38 MAPK pathway is thought to

be mainly responsible for synthetic functions and the secretion of cytokines in ASMCs, while the ERK-mediated pathway dominates in ASMC proliferation [3, 15, 16]. The role of JNK in the pathophysiology of asthma is somewhat more obscure [15], but experiments have shown that the JNK inhibitor SP600125 inhibits ASM hyperplasia and inflammatory cytokine release in mice chronically exposed to allergens [17].

The transcription factor nuclear factor-kappa B (NF-κB) plays an established role in airway inflammation [18]. On the other hand, its contribution to airway remodeling remains relatively unclear. The aim of this study is to examine how JNK, p38, and NF-κB contribute to ASMC proliferation.

Materials and methods

Cell culture

Normal human ASMCs (Normal Human Bronchial Smooth Muscle Cells; Lonza, Maryland, U.S.A.) and asthmatic human ASMCs (Diseased Bronchial Smooth Muscle Cells-Asthma; Lonza) were purchased from TAKARA BIO (Shiga, Japan). Experiments were carried out with normal and asthmatic human ASMCs derived from three independent subjects. The normal human ASMCs were derived from a 46-year-old Caucasian male (n1) and a 4-year-old black male (n2); the asthmatic human ASMCs were derived from a 27-year-old Caucasian male. Rat ASMCs obtained by a previously described method were also used [19]. The cells were cultured with a Smooth Muscle Cell Medium BulletKit™ (TAKARA BIO) or in Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, Missouri, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Moregate Biotech, Queensland, Australia), 50 U/ml of penicillin, and 50 mcg/ml of streptomycin (GIBCO, Life technologies, California, U.S.A.) at 37°C with 5% CO_2. Cells from the fourth to sixth passages were used. No ethics approval was sought for this study, as all of the experiments were performed *in vitro* using purchased human cells or rat cells stocked in our laboratory.

Proliferation assay (crystal violet assay)

ASMCs were plated in 96-well plates (Falcon®, Becton Dickinson, New Jersey, U.S.A.) at a density of 1×10^3 cells/well. After 24 hours starvation in 0.1% FBS, the cells were incubated in DMEM with 10% FBS in the presence or absence of JNK inhibitor SP600125 (Sigma-Aldrich) (10 μM), p38 inhibitor SB203580 (COSMO BIO, Tokyo, Japan) (10 μM), or NF-κB inhibitor sc-3060 (Santa Cruz Biotechnology, Inc. California, U.S.A.) (10 μg/ml). After six days the cell amounts were estimated by crystal violet assay [20]. In brief, the medium was removed from the 96-well culture plates and the cells in each well were washed with 200 μl of cold PBS and stained with 100 μl of an 0.5% crystal violet (Sigma-Aldrich) solution in 20% methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature for 10 minutes. The crystal violet solution was removed, the plate was washed with water until color no longer came off in the rinse, and the cells were dried overnight. The cells were solubilized the next day with 100 μl of 1% sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Ltd.) and the optical density of the plates was measured at 550 nm in a microplate absorbance reader (iMark™, Bio-Rad, California, U.S.A.).

The SP600125 and SB203580 were dissolved in dimethyl sulfoxide (DMSO), so DMSO was used as vehicle in the control experiments.

Proliferation assay (tritiated-thymidine incorporation)

ASMCs were plated in 96-well plates at a density of 5×10^3 cells/well and treated with each inhibitor for 2 days. Twenty-four hours after adding 1 μCi of tritiated thymidine, the cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA), harvested by filtration (UniFilter-96 GF/C, PerkinElmer Inc., Massachusetts, U.S.A.), and dried. The radioactivity of the incorporated tritiated thymidine was measured with a liquid scintillation counter (Topcount Liquid Scintillation Counter, PerkinElmer Inc.) after a soak in 20 μL of MicroScint™-20 (PerkinElmer Inc.).

C-jun N-terminal kinase inhibition by small-interference RNA (siRNA) transfection

SiRNA transfection was performed with Lipofectamine® RNAiMAX Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Briefly, ASMCs in 96-well plates were transfected with siRNA targeting JNK (Sig-

Table 1. Primer sets for real-time PCR analysis

Gene			Primer sequence
Human	Cyclin D1	Forward	5'-GACCATCCCCCTGACGGCGGAG-3'
		Reverse	5'-CGCACGTCGGTGGGTGTGC-3'
	p21	Forward	5'-TGATTAGCAGCGGAACAAG-3'
		Reverse	5'-AAACAGTCCAGGCCAGTATG-3'
	p27	Forward	5'-CGACCTGCAACCGACGATTCT-3'
		Reverse	5'-CCCCGCTCCACGTCAGTTCC-3'
	FGF1	Forward	5'-AACTCCTCTACTGTAGCAA-3'
		Reverse	5'-GGTGTTGTAATGGTTCTCCTCC-3'
	FGF2	Forward	5'-AGCGACCCTCACATCAAGCTACA-3'
		Reverse	5'-TGCCCAGTTCGTTTCAGTGCCA-3'
	TGF-β	Forward	5'-GCCCTGGACACCAACTATTGC-3'
		Reverse	5'-GCTGCACTTGCAGGAGCGCAC-3'
	CTGF	Forward	5'-CCGACTGGAAGACACGTTTGG-3'
		Reverse	5'-TCATGCCATGTCTCCGTACATCTT-3'
	PDGF-A	Forward	5'-CCTGCCCATTCGGAGGAAGAG-3'
		Reverse	5'-TTGGCCACCTTGACGCTGCG-3'
	PDGF-B	Forward	5'-GAAGGAGCCTGGGTTCCCTG-3'
		Reverse	5'-TTTCTCACCTGGACAGGTCG-3'
	EGF	Forward	5'-CAGCAGCACTGTGTGTGGGCA-3'
		Reverse	5'-ATCGGGTGAGGAACAACCGCT-3'
	JNK1	Forward	5'-CTGAAGCAGAAGCTCCACCA-3'
		Reverse	5'-CTGCACCTAAAGGAGAGGGC-3'
	JNK2	Forward	5'-AGATGCAGCAGTAAGTAGC-3'
		Reverse	5'-GTCGAGGCATCAAGACTGCT-3'
	G3PDH	Forward	5'-AGCAATGCCTCCTGCACCACCAAC-3'
		Reverse	5'-CCGCAGGGGGCATCCACAGTCT-3'
Rat	Cyclin D1	Forward	5'-AGTTGCTGCAATGGAACTG-3'
		Reverse	5'-GAAAGTGCGTTGTGCGGTAG-3'
	G3PDH	Forward	5'-ACGGGAAACCCATCACCATC-3'
		Reverse	5'-CCCTTCCACGATGCCAAAGT-3'

FGF; Fibroblast growth factor, TGF-β; Transforming growth factor-beta, CTGF; Connective tissue growth factor, PDGF; Platelet-derived growth factor, EGF; Epidermal growth factor, JNK; C-jun N-terminal kinase, G3PDH; Glyceraldehyde-3-phosophate dehydrogenase.

nalSilence® SAPK/JNK siRNA II #6233, Cell Signaling Technology, Inc.) using Lipofectamine® RNAiMAX Reagent for 48 hours. Control siRNA (Santa Cruz Biotechnology, Inc.) was used as a negative control. After 24 hours of starvation, the cells were stimulated by DMEM 10% FBS. After 3 days, cell proliferation was estimated by crystal violet assay. The efficiency of JNK inhibition by siRNA was examined by real-time reverse transcription polymerase chain reaction (RT-PCR) and western blotting. The primer sequences of JNK are shown in **Table 1**. The western blotting was carried out using JNK antibody (human/mouse/rat JNK pan specific mAb (Clone 252355) #MAB1387, R&D Systems, Minnesota, U.S.A.) at 1: 2500 and secondary antibody (biotinylated anti-mouse IgG, #BA20-00, Vector Laboratories, California, U.S.A.) at 1:800.

Annexin V and flow cytometric apoptosis analysis

An Annexin V-FITC apoptosis detection kit (Beckman Coulter, California, U.S.A.) was used to quantify the percentage of cells undergoing early apoptosis. The cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences, New Jersey, U.S.A.) and evaluated using Cell Quest software (BD Biosciences).

RNA isolation and quantitative real-time PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen, California, U.S.A.). RNA was reverse-transcribed into cDNA with SuperScript™ III Reverse Transcriptase (Invitrogen, Life technologies, California, U.S.A.). Quantitative real-time PCR was performed with specific primers (**Table 1**) and SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) using the Mini-Opticon™ Real-Time PCR Detection System (Bio-Rad). The target mRNA expression was established as relative units against the housekeeping gene glyceraldehyde-3-phosophate dehydrogenase (G3PDH).

Western blot analysis

The cell total proteins were extracted with RIPA buffer (150 mM NaCl (Wako Pure Chemical Industries, Ltd.), 1.0% NP-40 (Sigma-Aldrich), 50 mM Tris-HCl (Trizma® base; Sigma-Aldrich) pH 7.4, 0.5% Na-deoxycholate (Wako Pure Chemical Industries, Ltd.), 0.1% SDS (Wako Pure Chemical Industries, Ltd.), 2 mM EDTA (Wako Pure Chemical Industries, Ltd.), 50 mM NaF (Sigma-Aldrich), 0.2 mM Na_3VO_4 (Santa Cruz Biotechnology, Inc.), and protease inhibitor cocktail (Sigma-Aldrich). The protein was separated using SDS-PAGE, transferred to the PVDF membranes (Millipore Corporation, Massachusetts, U.S.A.), combined with cyclin D1

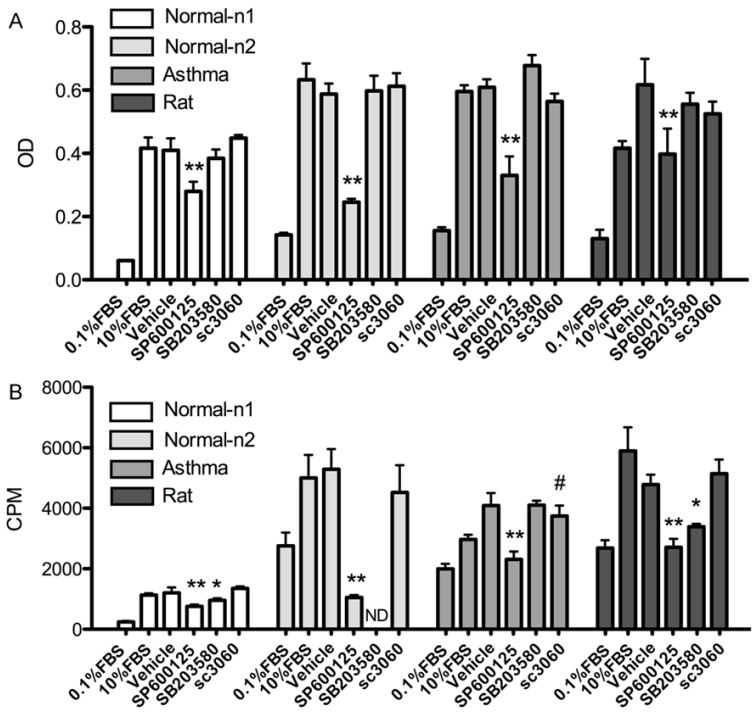

Figure 1. JNK inhibitor strongly suppresses airway smooth muscle cell proliferation, while NF-κB and p38 do not. Cell proliferation was assessed by crystal violet assay (A) and tritiated-thymidine incorporation (B). Normal-n1, normal human airway smooth muscle cells 1; Normal-n2, normal human airway smooth muscle cells 2; Asthma, asthmatic human airway smooth muscle cells; Rat, rat airway smooth muscle cells. SP600125, chemical inhibitor for JNK; SB203580, chemical inhibitor for p38; Sc-3060, chemical inhibitor for NF-κB. The vertical scales are optical density (OD) in panel (A) and the count per minute (CPM) in panel (B). Results are mean ± SD of at least triplicate wells and are representative of three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared to 10% FBS with vehicle (DMSO). $\#P < 0.05$ compared to 10% FBS alone. ND; not determined.

antibody (cyclin D1 antibody #2922, Cell Signaling Technology, Inc., Massachusetts, U.S.A.) at 1:2000, and incubated with secondary antibody (Amersham anti-rabbit IgG, biotinylated species-specific whole antibody #RPN1004, GE Healthcare UK Ltd., Buckinghamshire, England) at a 1:500 dilution. The membranes were then incubated with a VECSTAIN Elite ABC standard kit (Vector Laboratories) to amplify the signals. The light emitted with ECL Prime western blotting detection reagents (GE Healthcare) was exposed to X-ray film. The signal intensities were measured by analyzing developed film using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/). To control for loading differences, the protein levels were normalized against the levels of beta-actin protein detected using anti β-actin antibody (anti-actin [ACTN05 (C4)] antibody #ab3280, Abcam, Cambridge, England) at 1:2000.

Cell cycle analysis

After treatment, the cells were detached and fixed in cold 70% ethanol (Wako Pure Chemical Industries, Ltd.) overnight. The cells were then treated with 0.2 mg/mL RNase (Ribonuclease A #R65-13, Sigma-Aldrich) to remove RNA, and the DNA in the cells was stained with 20 μg/mL propidium iodide (PI) (#P4170, Sigma-Aldrich) for 30 minutes at room temperature. The samples were analyzed by a FACSCalibur (BD Bioscience Japan) flow cytometer and Cell Quest software.

Statistical analysis of results

All experiments were repeated at least three times. Data were presented as the mean ± standard deviation (SD) and analyzed by one-way ANOVA followed by Dunnett's post hoc test or unpaired t-test. *P* values of less than 0.05 were regarded as statistically significant. All statistical analysis was performed using GraphPad Prism 5 software (GraphPad, California, U.S.A.).

Results

Involvement of the JNK pathway in FBS-induced ASMC proliferation

The cell proliferation was assessed by crystal violet assay and tritiated-thymidine incorporation. The results of each assay are shown in **Figure 1A** and **1B**. In both assays, SP600125 significantly inhibited 10% FBS-induced cell proliferation in normal human ASMCs-n1, normal human ASMCs-n2, asthmatic human ASMCs, and rat ASMCs. As shown in **Figure 1B**, SB203580 inhibited DNA synthesis in normal human ASMCs-n1 and rat ASMCs, while sc-3060 promoted DNA synthesis in asthmatic human ASMCs. The cell amounts, however, remained unchanged in response to treatment by either SB203580 or sc-3060 (**Figure 1A**).

JNK siRNA transfection also significantly attenuated ASMC proliferation compared to control

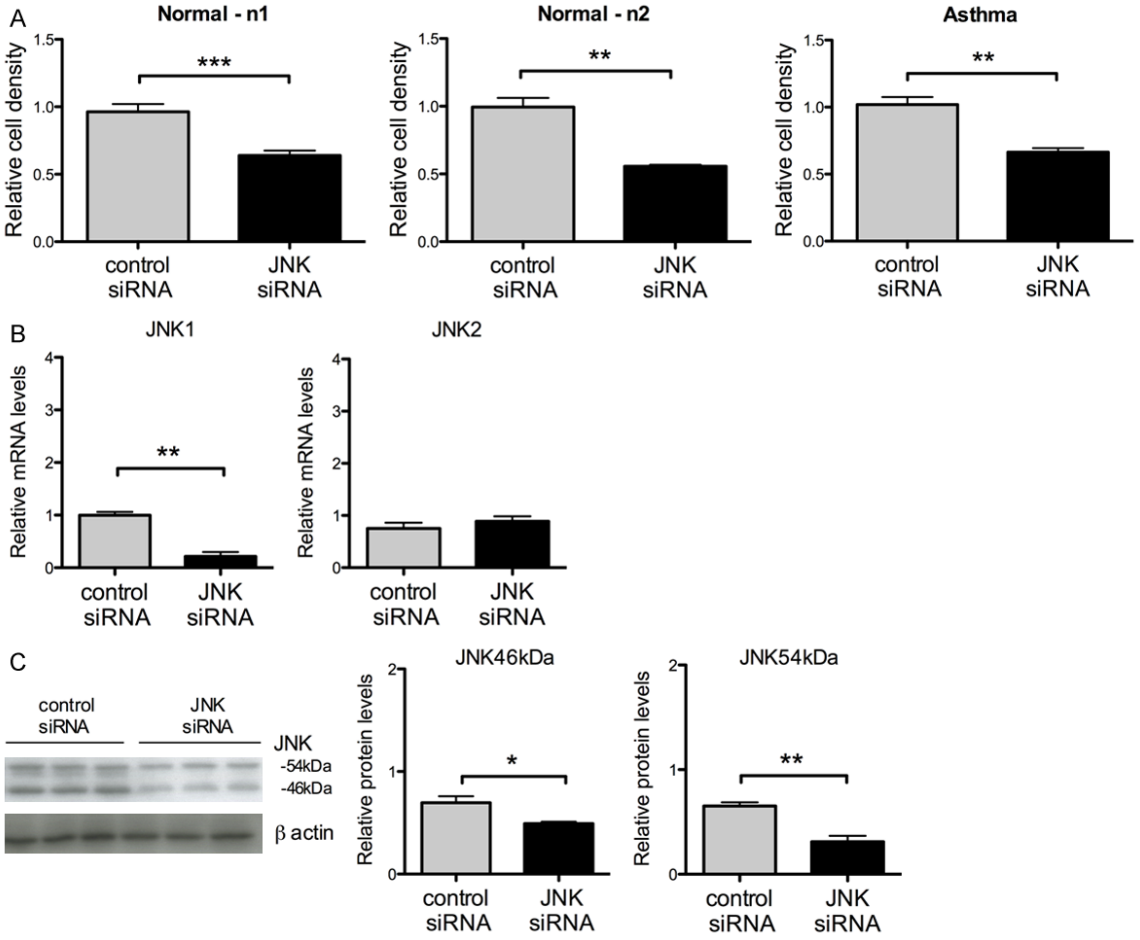

Figure 2. JNK siRNA suppresses airway smooth muscle cell proliferation. A. ASMC proliferation was assessed by crystal violet assay. Normal-n1, normal human airway smooth muscle cells 1; Normal-n2, normal human airway smooth muscle cells 2; Asthma, asthmatic human airway smooth muscle cells. B. The efficiency of JNK1 and JNK2 mRNA inhibition by siRNA was assessed by quantitative real-time RT-PCR (the representative results in Normal-n2 are shown). The data are normalized to the levels of the housekeeping gene G3PDH. C. The efficiency of JNK1 and JNK2 protein inhibition by siRNA was assessed by western blotting analysis (the representative results in Normal-n2 are shown). The left panels show the western blots and the middle and right panels show the signal intensities of the bands at JNK 46 kDa and JNK 54 kDa. The data are normalized to the levels of housekeeping protein β-actin. Results are mean ± SD of triplicate wells and are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

siRNA (**Figure 2A**). The JNK inhibition by siRNA was confirmed by quantitative real-time RT-PCR (**Figure 2B**) and western blotting analysis (**Figure 2C**). The mRNA levels of two ubiquitously expressed isoforms, JNK1 and JNK2, were measured. JNK1 mRNA expression was reduced to 18.86 ± 1.95% (normal human ASMCs-n1), 13.58 ± 1.02% (normal human ASMCs-n2), and 21.35 ± 8.47% (asthmatic human ASMCs). No inhibition of JNK2 mRNA was observed in any of the cell types. JNK siRNA also significantly inhibited the JNK protein levels for both the long (54 kDa) and short (46 kDa) variants in the western blot analysis. **Figure 2B** and **2C** show representative results of the PCR and western blotting in normal human ASMCs-n2.

Effects of JNK on ASMC apoptosis

An apoptosis assay was performed after 5 days treatment with SP600125 (10 μM). SP600125 brought about no change in the rate of apoptosis compared to vehicle (normal human ASMCs-n1, 0.04 ± 0.04% vs 0.01 ± 0.01%; normal human ASMCs-n2, 1.60 ± 1.68% vs 2.92 ± 2.76%; asthmatic human ASMCs, 0.09 ± 0.16% vs 0.15 ± 0.18%).

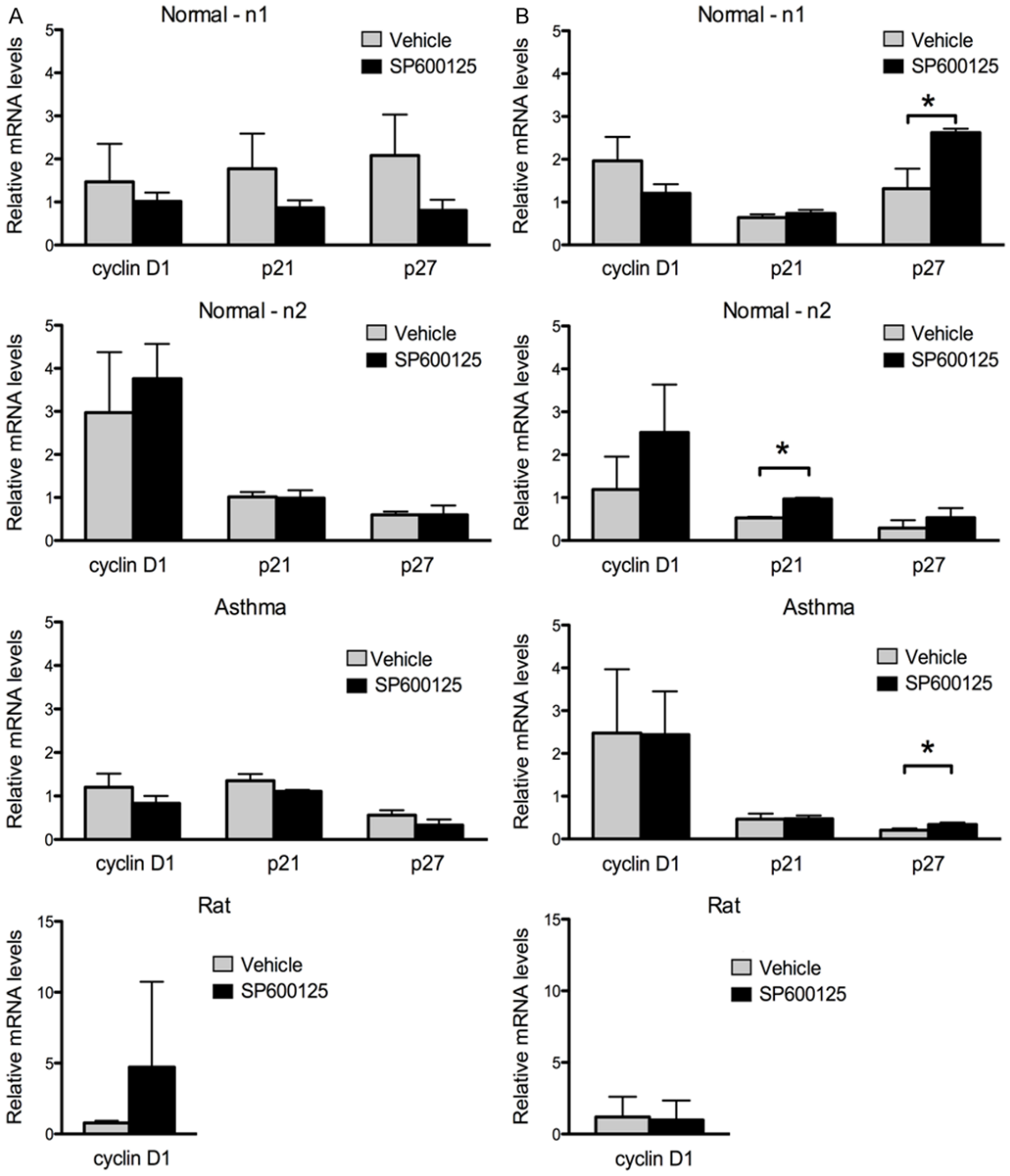

Figure 3. JNK inhibitor does not change the expression of cyclin D1, p21, or p27 mRNA. The results of quantitative real-time PCR at 6 hours (A) and 17 hours (B). Normal-n1, normal human airway smooth muscle cells 1; Normal-n2, normal human airway smooth muscle cells 2; Asthma, asthmatic human airway smooth muscle cells. Shaded bar, DMEM 10% fetal bovine serum (FBS) with vehicle (DMSO); closed bar, DMEM 10% FBS with SP600125, the chemical inhibitor for JNK. The data are normalized to the levels of the housekeeping gene G3PDH and expressed as fold changes relative to the value of starvation in 0.1% FBS. Results are mean ± SD of triplicate wells and are representative of three independent experiments. *$P < 0.05$.

Effect of JNK on the mRNA expression of cell cycle regulatory molecules

The mRNA was extracted from the ASMCs after 6 or 17 hours of treatment with SP600125 (10 μM). No significant changes were seen in cyclin D1, p21, or p27 mRNA levels at 6 hours (**Figure 3A**). The cyclin D1 mRNA levels were still unchanged after 17 hours of SP600125 treatment, but the p21 mRNA levels were increased

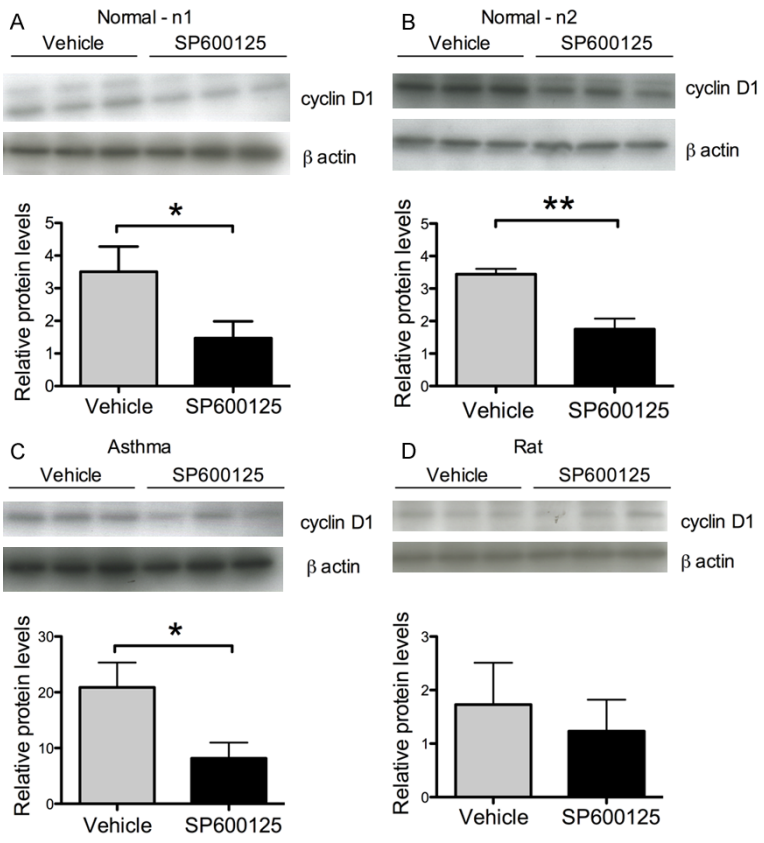

Figure 4. Cyclin D1 protein is decreased by the JNK inhibitor in various types of airway smooth muscle cell lines. A. Normal human airway smooth muscle cells 1. B. Normal human airway smooth muscle cells 2. C. Asthmatic human airway smooth muscle cells. D. Rat airway smooth muscle cells. Shaded bar, DMEM 10% fetal bovine serum (FBS) with vehicle (DMSO); closed bar, DMEM 10% FBS with SP600125, the chemical inhibitor for JNK. The upper panels show the western blots and the lower panels show the signal intensities of the bands. The data are normalized to the levels of housekeeping protein β-actin and expressed as fold changes relative to the value of starvation in 0.1% FBS. Results are mean ± SD of triplicate wells and are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$.

in normal human ASMCs-n2 and the p27 mRNA levels were increased in normal human ASMCs-n1 and asthmatic human ASMCs (**Figure 3B**).

Effect of JNK on cyclin D1 protein levels in ASMC proliferation

Cellular protein was extracted after 20 hours of treatment with SP600125 (10 μM). SP600125 significantly reduced cyclin D1 protein levels in normal human ASMCs-n1 (**Figure 4A**), normal human ASMCs-n2 (**Figure 4B**), and asthmatic human ASMCs (**Figure 4C**) compared to vehicle. The cyclin D1 protein levels in the rat ASMCs were not significantly changed by SP600125 treatment (**Figure 4D**).

Effect of JNK on mRNA expression of growth factors

SP600125 had no effect on the expression of any growth factor mRNA levels at 6 hours (**Figure 5A**) or 17 hours in either normal or asthmatic human ASMCs (**Figure 5B**).

Effect of JNK on cell cycle progression

A cell cycle analysis was performed after 24 hours of treatment with SP600125 (10 μM). Compared to vehicle, SP600125 significantly decreased the percentage of cells in the S phase (3.8 ± 0.2% vs 3.0 ± 0.4%) and G2/M phase (8.8 ± 0.6% vs 7.1 ± 0.8%) (**Figure 6**).

Discussion

The present study is the first to demonstrate that the JNK pathway takes part in human ASMC proliferation by upregulating cyclin D1 and promoting cell cycle progression.

The JNK subfamily contains ten isoforms of 46 or 54 kDa generated by alternative splicing of three genes (*JNK1*, *JNK2*, *JNK3*) [21]. The JNK inhibitor SP600125 was identified as a low-molecular-weight inhibitor of JNK1, -2, and -3 with a greater than 20-fold selectivity against the related MAPK, ERK and p38 pathways [22]. We also used JNK siRNA to confirm that the inhibition of ASMC proliferation was caused specifically by the suppression of the JNK pathway. JNK mediates cell apoptosis, cell proliferation, survival, and differentiation in response to pro-inflammatory cytokines and environmental stresses [23]. These various roles of JNKs have been attributed to the observation that JNKs activate different substrates based on specific stimulus, cell types, or temporal aspects [24]. JNK is confirmed to take part in airway remodeling [17, 25, 26], but it remains unclear whether it does so by attenuating cell apoptosis, promoting cell cycle progression, or increasing growth factor

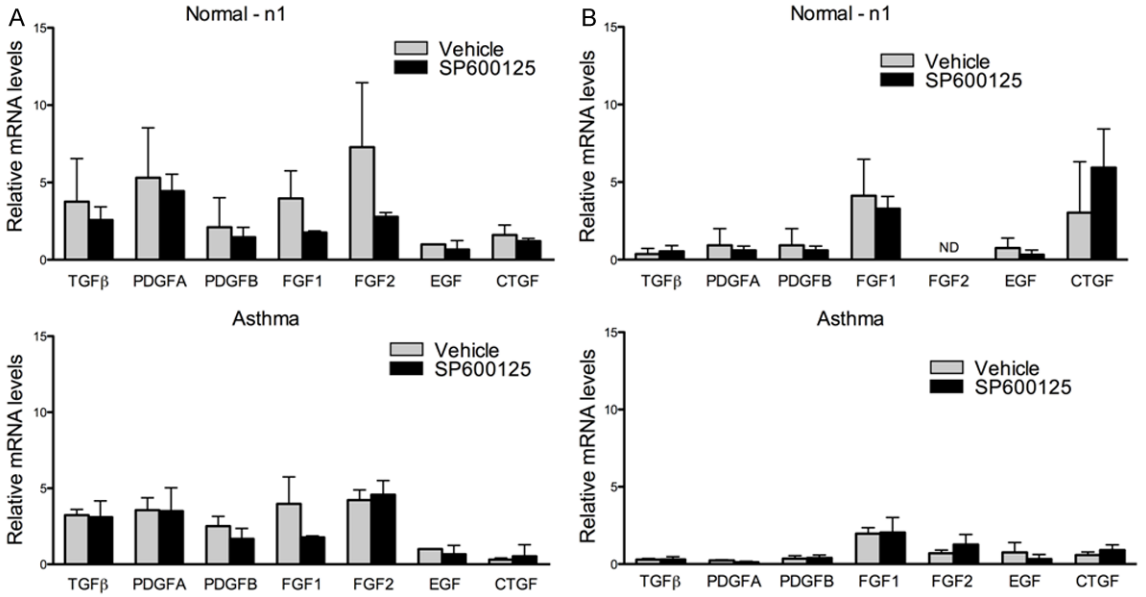

Figure 5. JNK inhibitor does not change the expressions of various growth factor mRNAs in airway smooth muscle cells. A. Growth factor m-RNA expression at 6 hours. B. Growth factor m-RNA expression at 17 hours. The results of quantitative real-time PCR in normal human bronchial airway smooth muscle cells 1 (Normal-n1) and asthmatic human bronchial airway smooth muscle cells (Asthma). Normal human airway smooth muscle cells 2 were not examined. Shaded bar, DMEM 10% fetal bovine serum (FBS) with vehicle (DMSO); closed bar, DMEM 10% FBS with SP600125, the chemical inhibitor for JNK. TGF-β, transforming growth factor-beta; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; CTGF, connective tissue growth factor. The data are normalized to the levels of the housekeeping gene G3PDH and expressed as fold changes relative to the value of starvation in 0.1% FBS. Results are mean ± SD of triplicate wells and are representative of three independent experiments. ND, not detected.

expression in ASMC proliferation. This study unraveled some of these unanswered questions. Our results indicate that the JNK pathway contributes to ASMC proliferation via the upregulation of cyclin D1 protein levels and driving cell cycle progression, at least in part. The absence of any significant change of cyclin D1 mRNA levels via JNK inhibition with SP600125 in our experiments suggests that JNK alters the post-translational regulation of cyclin D1 in ASMCs.

We hypothesized that the proliferative effect of JNK might also be caused by the inhibition of apoptosis. SP600125, however, had no observable influence on ASMC apoptosis. Our results suggest that ASMC proliferation via the JNK pathway is tied not with a decrease in the rate of apoptosis, but with an increase in the rate of proliferation.

ASMCs release several mediators such as cytokines, chemokines, and growth factors for themselves [2]. These mediators are believed to regulate ASMC synthetic and proliferative functions in both an autocrine and paracrine manner [27]. To investigate further, we assessed the possibility that JNK was capable of inducing ASMC proliferation by regulating the gene expression of growth factors. As it turned out, JNK exhibited no effects on the mRNA expressions of various growth factors.

P38 MAPK plays a key role in the secretion of pro-inflammatory cytokines from the ASMCs [28, 29]. Experiments with *in vivo* models have further underscored the importance of p38 MAPK in airway remodeling, together with airway inflammation and AHR [30, 31]. Our own experiments failed to demonstrate an involvement of p38 MAPK in human ASMC proliferation comparable to that in rat ASMCs, but this discrepancy may be attributable to a difference in stimuli or cell species. The contribution of the p38 MAPK pathway to the proliferation of human cultured ASMCs is actually mitogen-specific [32], and p38 MAPK has been found to negatively regulate transforming growth factor-beta (TGF-β)-stimulated human ASMC prolifera-

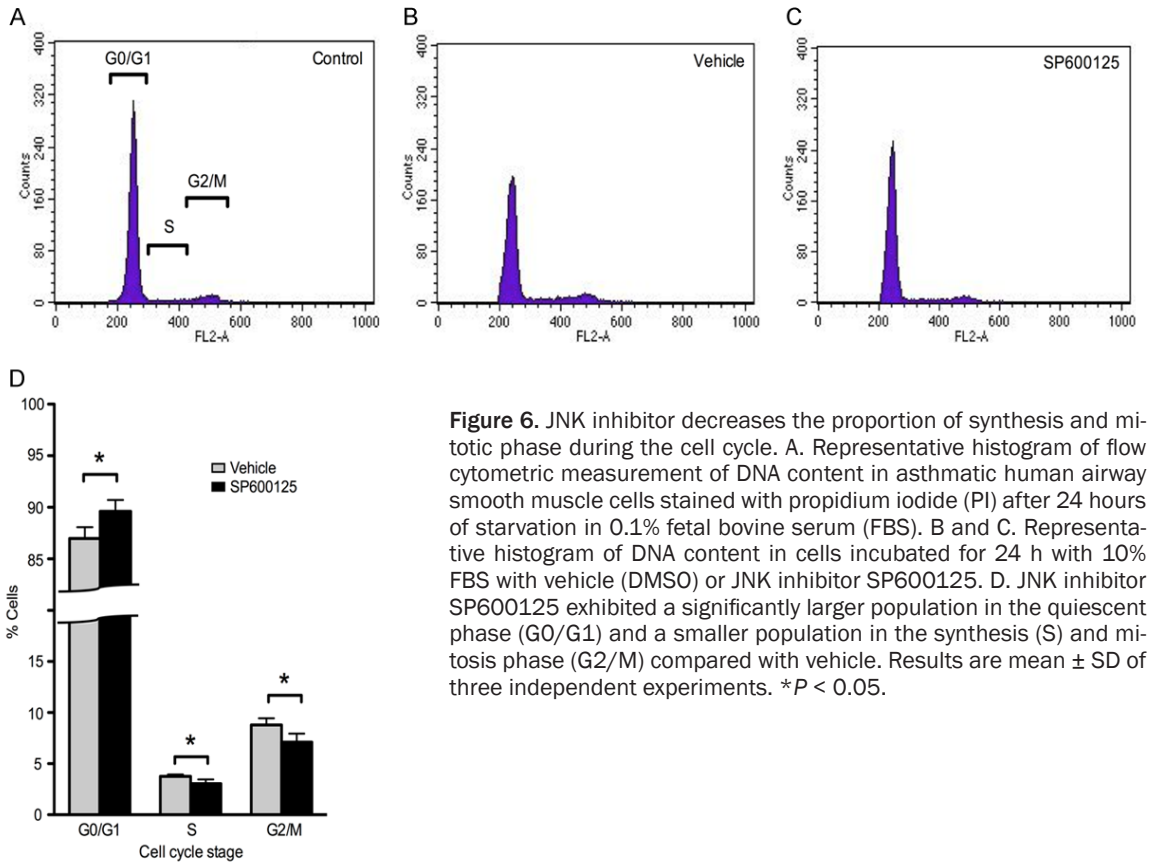

Figure 6. JNK inhibitor decreases the proportion of synthesis and mitotic phase during the cell cycle. A. Representative histogram of flow cytometric measurement of DNA content in asthmatic human airway smooth muscle cells stained with propidium iodide (PI) after 24 hours of starvation in 0.1% fetal bovine serum (FBS). B and C. Representative histogram of DNA content in cells incubated for 24 h with 10% FBS with vehicle (DMSO) or JNK inhibitor SP600125. D. JNK inhibitor SP600125 exhibited a significantly larger population in the quiescent phase (G0/G1) and a smaller population in the synthesis (S) and mitosis phase (G2/M) compared with vehicle. Results are mean ± SD of three independent experiments. $*P < 0.05$.

tion [33]. We chose FBS as an ASMC mitogen instead of specific substrate because we knew it would reflect physiological environments.

NF-κB is considered a master regulator of inflammatory responses, and its involvement in ASMC proliferation has been demonstrated *in vitro* [33, 34]. We therefore expected that the inhibition of NF-κB by sc-3060 would also suppress ASMC proliferation. Our results, however, failed to prove such an action.

Our study was limited by the small number of human-derived cell lines examined. The results were almost consistent among the human and rat cell lines in our study, but individual differences would have affected the results if we had used other human-derived cell lines. Further investigation to elucidate a universal role of JNK in ASMC proliferation will be necessary. In conclusion, the JNK pathway contributes to human ASMC proliferation, at least in part, via the upregulation of cyclin D1. JNK inhibition may become a novel target therapy for airway remodeling in asthma.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

ASM, Airway smooth muscle; ASMC, Airway smooth muscle cell; AHR, Airway hyperresponsiveness; JNK, C-jun N-terminal kinase; NF-κB, Nuclear factor-kappa B; PI3K, Phosphoinositide 3 kinase; ERK, Extracellular signal-regulated kinase; MAPK, Mitogen activated protein kinase; RT-PCR, Reverse transcription polymerase chain reaction; siRNA, Small interfering RNA; TGF-β, Transforming growth factor-beta; DMEM, Dulbecco modified eagle medium; FBS, Fetal bovine serum; DMSO, Dimethyl sulfoxide; G3PDH, Glyceraldehyde-3-phosophate dehydrogenase; PI, Propidium iodide.

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