Int J Clin Exp Pathol 2015;8(10):13753-13758
www.ijcep.com /ISSN:1936-2625/IJCEP0015096

Original Article

Effect of p38 mitogen-activate protein kinase on MUC5AC protein expression of bile duct epithelial cells in hepatolithiasis patients

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Received August 26, 2015; Accepted September 28, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: Primary hepatolithiasis is a common bile duct disease with benign nature but complicated mechanisms. Current studies have revealed its correlation with cytokine release by chronic inflammation, which also increased mucin (MUC) synthesis. This study investigated the role of p38 mitogen-activated protein kinase (MAPK) in regulating cytokine release and mucin synthesis, in an attempt to elucidate the role of p38 signaling molecule in the pathogenesis of hepatolithiasis. In human intrahepatic bile duct endothelial cells (HIBECs), lipoprotein (LPS) was used to induce the high expression of MUC. Small interference RNA (siRNA) was then used to silencing p38 gene expression. Cytokines including interleukin (IL)-1β and tumor necrosis factor (TNF)-α were measured, along with MUC5AC protein and mRNA expression assay. The interference of p38 gene expression inhibited the release of IL-1β and TNF-α in cultured cells. It also depressed both mRNA and protein levels of MUC5A. P38 MAPK signal pathway may be involved in the formation and progression of hepatolithiasis. This study provides potential new strategy for treating hepatolithiasis using p38 MAPK signal pathway as the drug target.

Keywords: Hepatolithiasis, lipoprotein, p38 MAPK, mucin

Introduction

Primary hepatolithiasis is a common bile duct disease in China and Far East regions [1]. It has complicated pathogenesis mechanism and may lead to severe consequences such as liver failure at the terminal stage. Pathological studies have confirmed that dilated intrahepatic bile duct calculus was accompanied with chronic hyper-proliferative inflammation, fibrosis of bile duct wall and hyperplasia of peripheral glands of the bile duct, but without obstruction of extrahepatic bile duct [2]. The abundantly secretion of mucus is also one pathological feature of bile ducts in hepatolithiasis patients, suggesting the role of mucin (MUC), which is the major component of mucus, in the formation of calculi [3]. One possible mechanism involves the secretion of MUC under the stimulation on bile duct epithelial cells for cytokine and inflammatory factor activation [4]. MUC is aggregated on the wall and cavity of bile duct, causing mechanic obstruction of bile duct and cholestasis, both of which facilitate the formation of calculi and aggravate local inflammatory response, for further potentiation of MUC production.

As one kind of intracellular signal molecule, p38 mitogen-activated protein kinase (MAPK) can be activated by a cascade of stress response including osmotic challenge, inflammatory cytokine, lipoprotein (LPS), ultraviolet and growth hormone shock, to exert pluripotent biological functions via downstream signals [5]. The activation of p38 has been detected in various hepatocyte injuries complicated with inflammation, such as the chemical liver injury [6], alcoholic or non-alcoholic fatty liver disease [7]. In general, the activation of p38 can aggravate inflammation. The production of mucin in intrahepatic bile duct calculi is also caused by inflammatory response. The effect of interference on p38 synthesis on the release of inflammatory cytokines and mucin synthesis, however, is still unclear.

Table 1. siRNA sequence

Name	Sequence (5'-3')	Bp
p38 siRNA sense	UGUGUAUCUGGUGACCCAUCUTdT	21
p38 siRNA anti-sense	AGAUGGGUCACCAGAUACACATdT	21
control siRNA sense	UUCUCCGAACGUGUCACGUUU	21
control siRNA non-sense	ACGUGACACGUUCGGAGAAUU	21

Table 2. Primer sequence for RT-PCR

Name	Sequence (5'-3')
MUC5AC-F	AGCGTGGAGAATGAGAAGTATG
MUC5AC-R	CATGCAGTTCGAGTAGTAGGTT
GAPDH-F	GGGAAACTGTGGCGTGAT
GAPDH-R	GAGTGGGTGTCGCTGTTGA

This study interfered synthesis of p38 by small interference RNA (siRNA) approach. The release level of IL-1β and TNF-α from cultured cells was examined, along with mRNA and protein levels of MUC5AC, in order to elucidate the role of p38 signal molecule in pathogenesis of hepatolithiasis.

Materials and methods

Cell culture and transfection

Human intrahepatic bile duct epithelial cell (HIBEC) was provided by Mingshan Biotech (Guangzhou, China) and was cultured in DMEF-F12 medium containing fetal bovine serum (FBS, Hyclone, UT, US). Cells with confluence between 30%~50% were seeded into 24-well plate. Synthesized siRNA (**Table 1** for sequences, produced by Sigma, Hong Kong) was prepared as previously documented [8] and was mixed in Opti-MEM medium (Invitrogen, CA, US), along with medium containing Lipofectamine 2000 (Invitrogen, CA, US). After 20-min incubation, the mixture was added into the plate. The transfection efficiency was quantified under a fluorescent microscope. 24 hours after transfection, all cells were given normal or LPS (100 mM, Sigma, Hong Kong) containing medium for induction of inflammation.

Western blotting

Cultured cells were collected and homogenized with RIPA lysis buffer (1 mM) for iced incubation (15 min). After centrifugation (14 000 rpm for 15 min), supernatants were collected for protein quantification using BCA method. After denature in boiled water for 5 min, proteins were separated by SDS-PAGE, and were transferred to PVDF membrane under an electrical field. Primary antibodies, including anti-Mucin 5AC and anti-p38 (1:1 000, Abcam, Hong Kong) were added for overnight incubation. On the next day, the membrane was washed by TBST, followed by rabbit anti-mouse IgG (H+L) (Proteintech, Wuhan, China) incubation for 2 hours. The membrane was then developed by ECL method.

Enzyme-linked immunosorbent assay (ELISA)

Using ELISA kits for human IL-1β and TNF-α (RayBio, GA, US), we measured cytokine levels from transfected HIBEC cells. In brief, standards and diluted samples were added into 96-well plate. After incubation for 2.5 hours, the supernatants were discarded, followed by washing and addition of biotin-labelled antibody (0.1 mL each well). The plate was rinsed after 1 hour, and was added with HRP-Strptavidin reagents for 45-min incubation. The chromogenic substrate (TMB) was added for dark incubation. The reaction was quenched by stopping buffer after 30 min. The plate was loaded onto a microplate reader for absorbance value at 450 nm.

Real-time quantitative PCR

Total RNA was extracted from collected cells in 1 mL Trizol reagents (Invitrogen, CA, US). After complete lysis of tissues, 0.2 mL chloroform was added and mixed for extracting RNA. After 15-min incubation and 10 000 g centrifugation, the supernatant was saved and precipitated in 0.5 mL isopropanol. RNA was precipitated under room temperature and collected by centrifugation (10 000 g, 10 min), followed by washing in 75% ethanol and air drying. RNA was re-suspended in 15 μL DEPC-treated water.

Using specific primers for MUC5AC as previously reported [9] (**Table 2**, Sigma, Hong Kong), PCR amplification was carried out using the following conditions: 50°C for 30 min and 95°C pre-denature for 5 min, followed by 30 cycles each containing 95°C denature for 30 sec, 55°C annealing for 30 sec and 72°C elongation for 50 sec. The reaction ended with 72°C elongation for 10 min. The relative expression of the

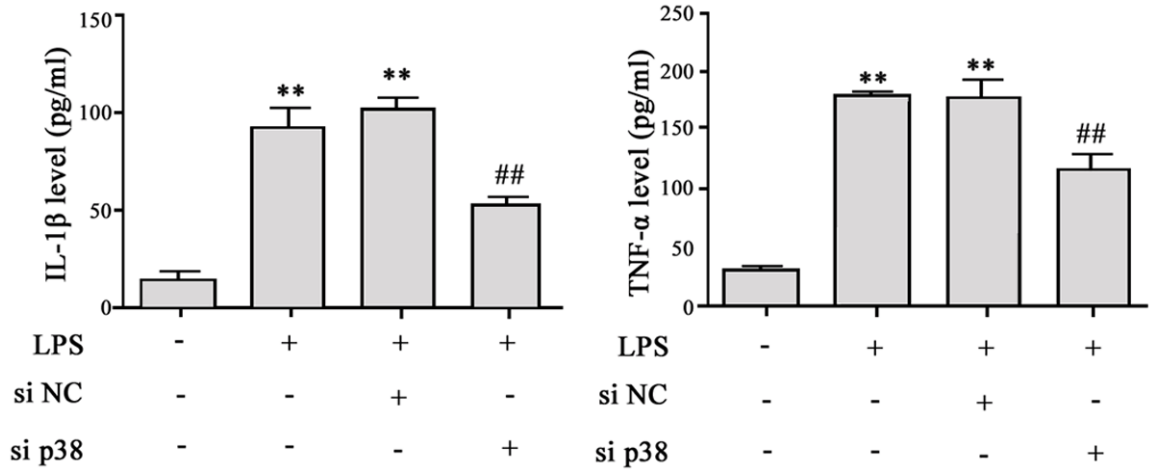

Figure 1. IL-1β (left) and TNF-α (right) levels in cultured medium of HIBECs. **P<0.05 compared to normal control group; ##P<0.05 compared to siRNA control (si NC) group.

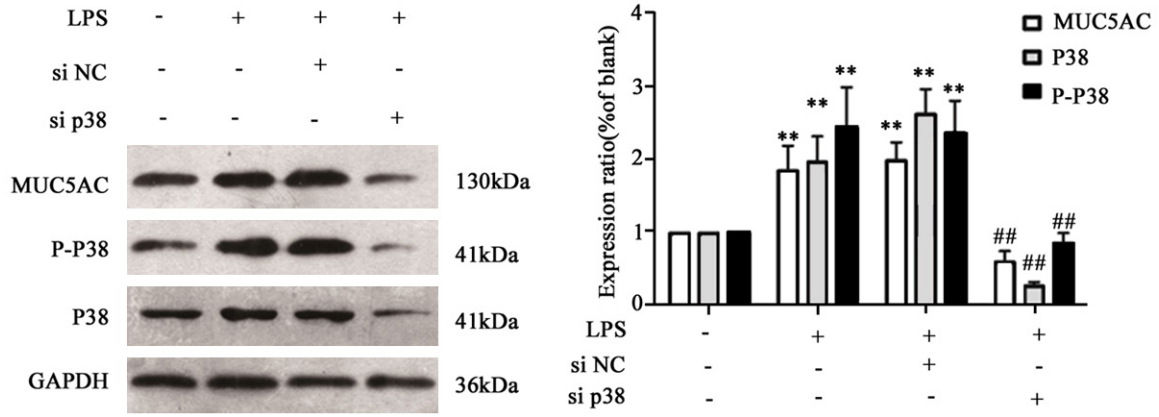

Figure 2. P38 phosphorylation (left) and MUC5AC expression (right) of cells. **P<0.05 compared to normal control group; ##P<0.05 compared to siRNA control (si NC) group.

target genes was determined by $2^{-\Delta\Delta Ct}$ method using GAPDH as the reference.

Statistical analysis

SPSS 10.0 software was used to collect all data, which were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to perform comparisons among all groups, followed by SNK-Q test to compare means between two groups.

Results

Cytokine release from HIBECs

The stimulus of LPS can activate the synthesis and release of IL-1β and TNF-α from HIBECs (**Figure 1**). The introduction of siRNA significantly depressed the release of both cytokines when compared to siRNA control and LPS groups (P<0.05, **Figure 1**).

MUC5AC and phosphorylated p38 expression

Multiple inflammatory related signal pathways can induce the elevated synthesis of MUC5AC [10]. Meanwhile the activation of p38 can induce further downstream signal pathways such as NF-κB [11]. To investigate the correlation between p38 expression and MUC5AC synthesis, we found that LPS treatment can activate the phosphorylation of p38, accompanied with elevated MUC5AC expression (**Figure 2**). The interference of p38 caused depressed MUC5AC level (**Figure 2**, P<0.05). These results

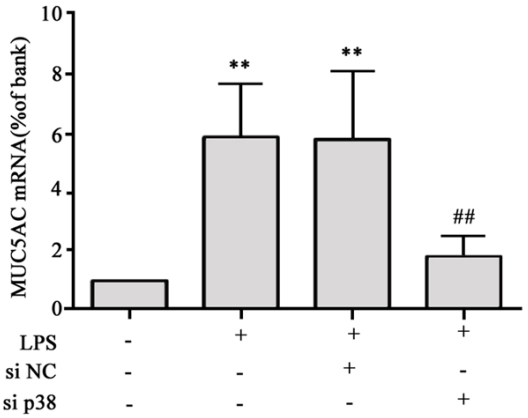

Figure 3. MUC5AC mRNA level. **P<0.05 compared to normal control group; ##P<0.05 compared to siRNA control (si NC) group.

suggested the potential involvement of p38 signal pathway in LPS-induced MUC5AC overexpression.

MUC5AC mRNA level

Consistent results have been obtained from mRNA of MUC5AC, as quantitative RT-PCR showed significantly suppressed MUC5AC mRNA level after the application of p38 siRNA (P<0.05, **Figure 3**).

Discussion

As a common surgical complication, intrahepatic bile duct calculi, or hepatolithiasis, have unclear pathogenesis yet. Mucin is a type of high-molecular weight glycoprotein to coat or protect epithelial cells [12]. Past studies have revealed mucin as one important factor in bile duct calculi, as the intrahepatic bile duct disease is often accompanied with abnormal secretion of mucin. It has now widely accepted the over-secretion of MUC played an important role in the formation of intrahepatic bile duct calculi [13]. Among various subtypes of MUC, the elevation of MUC2 and MUC5AC in hepatolithiasis was evident [14].

The mechanism of endothelial hyperplasia and MUC over-secretion, however, still remain unclear. More and more evidences supported the participation of inflammation in those processes [15]. Related pathological processes may involve the aggregation of abundant MUC in the inner wall or the cavity of bile duct, causing mechanical bile duct obstruction and cholestasis, both of which may further aggravate the condition of inflammatory response. Therefore, the inhibition of inflammatory factor and cytokine release and related MUC production, has the potency to become the new drug target.

Early clinical observations have found the occurrence of bile duct inflammation accompanied with calculi [16]. Other scholars also demonstrated the correlation between inflammation-induced hyperplasia of bile duct endothelial cells and the pathogenesis of intrahepatic bile duct calculi. In such chronic inflammation of bile duct, the re-formation of bile duct epithelial cells increases, along with over-secretion of MUC into the cavity. Similar process occurs in the bronchial epithelial cells, whose IL-1β induces MUC5AC secretion via cAMP-PKA signal pathway [17]. The over-expression of MUC5AC has also been suggested to be related with MAPK signal pathway [18]. Early studies have demonstrated the activation of MUC2 and MUC5AC expression by LPS via up-regulating TNF-α and protein kinase C signal pathway in mouse gall bladder epithelial cells [19]. In bacterial infection, LPS, as the major component of bacterial cell wall, can stimulate the release of multiple pro-inflammatory cytokines and factors via stimulating HIBECs, which were also induced to secrete abundant MUC [20].

As one important member of MAPK family, p38 is expressed in various cells and exerts certain pro-inflammatory roles [21, 22]. Its role in regulating MUC5AC in intrahepatic bile duct calculi, however, remained unclear. This study thus investigated if the inhibition of p38 may regulate MUC5AC secretion, for further treating intrahepatic bile duct calculi.

HIBEC can express abundant of MUC and thus mimics the formation of intrahepatic bile duct calculi [23]. We thus selected HIBEC as the drug target. As previously reported [24], LPS can induce the occurrence of inflammation, which is accompanied with MUC over-expression in HIBEC, therefore mimicking the *in vivo* formation of intrahepatic bile duct calculi. siRNA, with 20~24 nt length, can specifically bind with target mRNA by complementary base paring, and induce the post-transcriptional gene silencing under the direction of Dicer enzyme, thus down-regulating the level of target protein and further downstream signal pathways and activity of actors [25]. This study

utilized siRNA to inhibit the expression of p38, thus observing the role of p38 in calculi formation. We used HIBEC with the help of LPS induction to elevate MUC5AC expression. Our results found that LPS induced the over-expression of MUC5AC via p38 signal pathway in HIBECs. The transfection by p38-siRNA can significantly decrease the level of IL-1β and TNF-α in culture medium, plus the expression of MUC5AC. Our results collectively suggest the participation of p38 MAPK signal pathway in the formation and progression of intrahepatic bile duct calculi, and provide potential novel strategy for treating patients using p38-MAPK as the drug target.

Acknowledgements

Research supported by the Medical Scientific Research Foundation of Guangdong Province, China (Grant No. A2013254).

Disclosure of conflict of interest

None.

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