Original Article

Alpha mangostin Inhibits Hepatic Stellate Cells Activation Through TGF-β/Smad and Akt Signaling Pathways: An in vitro Study in LX2

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Abstract

Background Alpha mangostin has been reported to have activity for the treatment of liver fibrosis in the rats. However, the mechanisms of action are poorly understood. This study was aimed to investigate the effect of alpha mangostin on hepatic stellate cells (HSC) activation and proliferation through TGF-β/Smad and Akt signaling pathways.

Methods Immortalized HSC, LX2 cells, were incubated with TGF-β with or without alpha mangostin (5 or 10 μM). Sorafenib 10 µM was used as positive control. LX2 viability was counted using trypan blue exclusion method. The effect of alpha mangostin on TGF-β concentrations, and the expressions of proliferation and fibrogenic markers were evaluated.

Results Alpha mangostin treatment resulted in a reduced proliferation of HSC, decreased Ki-67 and p-Akt expressions. These findings were followed with decreased concentrations of TGF-β in the medium of cells treated with alpha mangostin, decreased expressions of COL1A1, TIMP1, PAI1, α-SMA, and p-Smad3 as fibrogenic markers. These effects were shown to be dose-dependent.

Conclusions Alpha mangostin inhibits hepatic stellate cells proliferation and activation through TGF-β/Smad and Akt signaling pathways in dose dependent manner.

Key words

Alpha mangostin - hepatic stellate cells - TGF-β - Smad - Akt

Introduction

It is well known that HSC activation plays a key role in the development of liver fibrosis [1] [2]. HSC persists quiescent in normal liver and serves as retinoid storage deposit. It also maintains the balance of basal membrane matrix. Many in vitro and in vivo studies represent chronic liver injuries induce HSC activation. HSC is the principal source of extracellular matrix
(ECM) during chronic liver injury. Under activation, HSC undergo a transformation from a quiescent to an active phenotype. Activation of HSC characterized by increased cell proliferation, enhanced matrix production and expressions of a number of proliferative and pro-fibrogenic cytokines and their receptors [3] [4] [5] [6].

TGF-β plays an important role in HSC activation and proliferation through Smad and non Smad pathways. Phosphorylation of Smad3 by type I TGF-β receptor is a crucial step in HSC activation. Phosphorylated Smad3 (pSmad3) is the most critical transcription factor for the induction of the fibrogenic genes in response to TGFβ1 signaling in HSC activation and closely correlated with increased α-smooth muscle actin (α-SMA) and collagen type I expression [4] [5] [7] [8].

There are many genes involves in the activation of TGF-β non Smad pathways, such as ERK1/2, Akt and Rho-A [9]. Among these genes, Akt has been reported to be an excellent marker for cell proliferation [10]. TGF-β inhibits ECM degradation by suppressing matrix metalloproteinase (MMP) and promoting its natural inhibitor, tissue inhibitor of metalloproteinase (TIMP). Markers of extracellular matrix, TIMP-1 and PAI-1 are expressed by hepatic stellate cells in response to inflammation in the liver. Mostly, these markers are involved in PI3K/Akt through crosstalk with Smad 2/3 and affect the transcription level in nucleus [11] [12].

Alpha mangostin, a xanthone derivates, is the lead active compound found in the tropical fruit Garcinia mangostana Linn. It has been reported that alpha mangostin has antifibrotic activity and decreased TGF-β levels. It also has antiproliferative effect in tumor cells [13]. However the mechanism of actions supported these findings were poorly explained. In the present study we investigated the activity of alpha mangostin in HSC activation and proliferation through TGF-β/Smad and Akt pathways.

Materials and Methods

Materials

Immortalized human hepatic stellate cells, LX-2 were purchased from Millipore (USA, Cat No.SCC064). Sorafenib and alfa mangostin were obtained from Santa Cruz Biotechnology (USA). Human TGF-β were purchased from Wako Pure Industries (Japan), while dimethylsulfoxide (DMSO) were from Sigma Aldrich (Singapore). DMEM-high glucose, FBS-heat inactivated were from Hi-Media (India), while penicillin/streptomycin and fungizone were from Biowest (USA). MTS Assay kit were purchased from Promega (USA). High pure RNA isolation kit, Transcriptor First Strand cDNA synthesis kit, FastStart DNA master SYBR green I were purchased from Roche (USA). Cell lysis buffer, TGF-β ELISA (enzyme-linked immunoassay) kit and Coomassie Plus (Bradford) assay kit were obtained from Invitrogen (USA). Primers were purchased from First Base (Singapore). Antibody alpha smooth muscle actin (PA5-19465) were from Thermo Fisher (USA), antibody β-actin (13E5) Rabbit mAB, Anti-rabbit IgG, HRP-linked Antibody, Smad3. Phospo-Smad3 (Ser 423/425) (CA25A9) rabbit mAb were obtained from Cell Signaling Technology (USA).

LX2 culture

LX2 was cultured and maintained at 37°C in a 5% CO₂ on Dubelcco’s modified Eagle’s Medium (DMEM)-high glucose supplemented with 10% heat inactivated fetal bovine serum (FBS), 10 U/L penicillin, 100 μg/mL streptomycin and 2.5 μg/mL fungizone.

MTS assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay was done to determine cytotoxicity concentration 50% (CC50) of sorafenib and alpha mangostin on LX-2 cells. Cells were seeded at 1 000 cells per well in a 96-well plate and incubated for 24 h in a CO₂ incubator. Afterwards, cells were treated with sorafenib or alpha mangostin at concentrations of 2, 5, 10, 20, 40, 80 and 100 µM for 24 h. Afterwards, the medium were discarded and replaced with a fresh 100 µL medium. Then, 20 µL of MTS solution were added to the medium and incubated for 2 h. Optical density (OD) was measured at 490 nm using microplate reader. CC50 of sorafenib and alpha mangostin were used as a basis to determine dose of alpha mangostin for LX-2 cells treatment.

Cell treatment

Cells were divided into five groups and seeded at 2×10⁶ for each group in 10-cm culture dish as follows: the first group was the untreated cells, while the other four groups were induced by 2 ng/mL TGF-β for 24 h. Afterwards the medium were changed. The first group remains with medium only. To the second group, TGF-β 2 ng/ml were applied. To the remaining 3 groups, the treatment applied were: TGF-β 2 ng/ml + sorafenib 10 µM, TGF-β 2 ng/ml + alpha mangostin 5 µM and TGF-β 2 ng/ml + alpha mangostin 10 µM, respectively. After 24 hour treatment, the cells were harvested and subjected for analysis of cell viability, RNA and protein isolation. Experiments were done in four different times in duplicate.

Cell viability examination using trypsin blue exclusion method

Cell harvested using trypsin EDTA and stained with trypsin blue. Cell viability were counted using hemocytometer.

qRT PCR analysis
RNA isolated from 10^6 cells using High Pure Isolation RNA kit (Roche). From 1 μg of total RNA template, cDNA were synthesized using Transcriptor First Strand cDNA Synthesis kit (Roche) in accordance to the manufacturer’s protocol. mRNA expression of COL1A1, TIMP1, PAI1 and Ki67 were analyzed using qRT-PCR. Quantification cycle (Cq) calculated using software. Cq data proceeded using Livak method to gain level of expressions. We used β-actin as housekeeping gene. The primer sequence used in this study are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
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<tbody>
<tr>
<td>COL1A1</td>
<td>Forward: AGGTCCCCCTGGAAAGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATCCTCGAGCACCCTGA</td>
</tr>
<tr>
<td>Ki67</td>
<td>Forward: TCCTTTGTGGGCACCTAAGCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGAATGTTGAGGTCCTTTGATG</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Forward: AGGTCCCCCTGGAAAGAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATCCTCGAGCACCCTGA</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Forward: ACAAGTTCAACTATAGGTTCACGGC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGAACACTGCTGACATGTCCGTCATTCCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: GCCATCGTGATGGAATCCG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGGAAGGGTGACAGCGA</td>
</tr>
</tbody>
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### Western blot analysis
Total protein was extracted from cells using cell lysis buffer solution (Invitrogen). Protein (50 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes that were sequentially blocked with defatted milk. The membrane was incubated with the primary antibody for α-SMA, pSmad3, Smad3, pAkt, Akt and β-actin at 4°C overnight. After washing with TBST, blots were then incubated with secondary antibodies at room temperature for 1 h. The specific protein bands were developed using a chemiluminescent substrate and imaged using a gel scanner. β-actin was used as the internal control.

### ELISA
TGF-β1 in the cell medium was measured using Human TGF-β1 ELISA kit (Invitrogen) in accordance to the protocol. TGF-β1 levels were measured in 450 nm using microplate reader.

### Statistical analysis
Results were presented as mean ± SD. Statistical analysis was performed using One-way ANOVA analysis. Statistical significance was determined at the level of p<0.05.

### Results

**Cytotoxicity concentrations of alfa mangostin and sorafenib**
To establish the dose used in treatment, we determine cytotoxicity concentration 50% (CC50) of alpha mangostin and sorafenib in LX2. As shown in Fig. 1 demonstrates the CC50 of alpha mangostin (a) in LX2 was 10.77 μM, while CC50 of sorafenib (b) was 10.56 μM. Therefore, we used sorafenib at 10 μM, and alfa mangostin at 5 μM and 10 μM.

![Fig. 1](https://www.thieme-connect.de/media/drugres/201803/10-1055-s-0043-119074-i2017-07-1446-0001.jpg)

**Fig. 1** Percentage of cell viability over control after 24 h treatment of LX-2 cells with alpha mangostin a and Sorafenib b at 2, 5, 10, 20, 40, 80 and 100 μM. Cytotoxicity concentrations 50% were extrapolated using equation of log dose versus percentage viability over control.
Cell proliferation

TGF-β induction resulted in a significant increase of LX2 proliferations as compared to untreated group. A 2 ng/ml TGF-β concentration was chosen based on previous studies [8]. [Fig. 2] shows that 2 ng/ml TGF-β concentration significantly promote LX2 proliferation as compared to untreated group (a), increased the expression of mRNA Ki-67 (b) and p-Akt (c) whereas alpha mangostin treatment significantly suppressed LX2 proliferation in dose dependent manner.

Alpha mangostin in both dosages showed lower antiproliferative effect as compared to positive control.

Alpha mangostin decreased TGF-β1 concentrations in LX2 culture medium

[Fig. 3] shows that induction with 2 ng/ml TGF-β, significantly increased TGF-β1 concentrations in the medium compared to untreated group. Alpha mangostin could inhibit TGF-β1 level significantly in a dose dependent manner, but still in lower activity as compared to positive control.

Alpha mangostin decreased expression of profibrogenic marker COL1A1, TIMP1, α-SMA and pSmad3

[Fig. 4a-c] shows that induction with 2 ng/ml TGF-β significantly increased COL1A1, TIMP1 and PAI1 mRNA expressions. Both dosages of alpha mangostin significantly suppressed the expression those profibrogenic markers. [Fig. 5a, b] shows that induction with 2 ng/ml TGF-β significantly increased α-SMA and pSmad3 protein expressions. Alpha mangostin in both dosages significantly suppressed those profibrogenic markers expressions.
Discussion

Previous report have demonstrated that alpha mangostin had antifibrotic activity in in vivo study [13], however the exact mechanism of action was still unknown. TGF-β/Smad and Akt signaling are the major signaling pathways that reflects fibrogenesis. [4] [5] [8]. Therefore, we are investigating these pathways to confirm whether antifibrotic activity of alpha mangostin was exerted via these pathways.

TGF-β has been confirmed as major pro-fibrogenic cytokine that plays a key role in HSC activation and proliferation [4] [6]. Previous study by Kang et al. (2013) and Zhu et al. (2014) showed that induction by TGF-β increase the HSC proliferation and expression of pro-fibrogenic marker [14] [15]. In the present study we confirmed that induction with TGF-β increased the HSC proliferation up to 2-folds compared with untreated cells. These findings were in line with the increase of Ki67, a marker for proliferation. Study by Zhang et al. (2010) showed that induction of TGF-β increased expression of Ki 67 in rabbit cornea cells [16]. Our present study demonstrated significant increase of mRNA Ki 67 expression after induction with TGF-β 2 ng/mL.

It also has been known that PI3K/Akt plays a role in myofibroblast proliferation. Previous study by Son et al. (2009) showed that inhibition of PI3K/Akt pathways decreased cell proliferation [10]. In our study we demonstrated that TGF-β induction affected PI3K/Akt pathways by increasing phosphorylation of Akt. Thus, we conclude that TGF-β has a major activity in increasing HSC proliferation.

The TGF-β/Smad pathway seems to be the major pathway responsible in HSC activation. Many previous study demonstrated that HSC activation correlated with increasing of phosphorylation of R-Smad2/3. Phosphorylated Smad2/3 in collaboration with CoSmad 4 transported into nucleus and acts as transcription factor for pro-fibrogenic marker α-SMA and COL1A1 [4] [5] [6]. In our study, we confirmed that induction by TGF-β increase phosphorylation of Smad3 significantly. These findings were in line with increasing of pro-fibrogenic marker expressions α-SMA and COL1A1.

TGF-β also promotes fibrogenesis by decreasing Matrix Metalloproteinase (MMPs) and promoting its natural inhibitor Tissue Inhibitor of Matrix Metalloproteinase (TIMPs) [11] [17]. In the present study, we confirmed that induction of TGF-β significantly increased TIMP-1 expression. Thus we confirmed that our model of TGF-β induction was successful, activation of HSC was markedly occurred, as all the pro-fibrogenic markers of HSC activation is significantly increased. This results were in accordance with previous study by Tang et al. (2012), Shi et al. (2006), and Kang et al. (2013) showed a significant increase in α-SMA, pSmad3 and Col1A1 expression on LX2 cell induced by TGF-β [15] [18] [19].

Our study showed that alpha mangostin supressed the proliferation of HSC under stimulation of TGF-β. Furthermore, we confirmed that there were marked increased concentrations of TGF-β in culture medium stimulated by TGF-β. Our result is in agreement to the study done by Dumont et al. that showed after cells lose the sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling may promote cell motility [20]. Alpha mangostin significantly decreased concentrations of TGF-β in culture medium. The effect appears to be dose dependent. This result was expected due to the activation of autocrine signaling, as showed by the result, the level of TGF-β increased by four fold.

The examination of signal molecule in TGF-β/Smad pathway showed that alpha mangostin decreased Smad3 phosphorylation during HSC activation. Previous study demonstrated that inhibition of Smad3 phosphorylation decreased ECM production in fibrogenesis [18] [19]. Our study confirmed that alpha mangostin inhibits Smad3 phosphorylation, thus inhibiting the downstream pathway resulted in decreasing pro-fibrogenic marker expression TIMP-1, COL1A1 and α-SMA.

In our study, we used sorafenib as positive control. Sorafenib is a multikinase inhibitor that has been studied as antifibrotic. Sorafenib inhibits receptor kinase phosphorylation and its downstream pathways [21] [22]. Previous studies had shown that sorafenib inhibits TGF-β Smad signaling and Akt signaling [23] [24] [25]. Treatment with sorafenib in experimental hepatic fibrosis also showed that extracellular matrix marker such as TIMP-1 could also be decreased [26].

Our study showed that alpha mangostin inhibits of TGF-β/Smad and Akt pathways at a comparably lower levels than positive control, as shown by its ability to inhibit HSC proliferation and activation.

Alpha mangostin tend to reverse the effect of TGF-β back to untreated condition, unlike that of positive control that showed decreased of TIMP-1 and p-Akt expressions below untreated condition.
In conclusion, alpha mangostin inhibits activation of HSC as shown by the decrease of profibrogenic marker expression and exerts antiproliferative activity through the inhibition of TGF-β/Smad and Akt pathways. Therefore, this study suggesting the possibility of using alpha mangostin as a potential candidate in the treatment of liver fibrosis.

Conflict of Interest

The authors declares no conflict of interests.

* equal contribution

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