

THE EFFECT OF ALPHA-MANGOSTIN ON TRANSFORMING GROWTH FACTOR BETA 1 (TGF- β 1) AND MATRIX METALLOPROTEINASE-3 EXPRESSION IN TGF- β -INDUCED HEPATIC STELLATE CELLS

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ABSTRACT

Objective: Alpha-mangostin (α -MG) has been shown to possess antifibrotic effects. However, the specific mechanism of action of this compound remains poorly understood. Therefore, the aim of this study was to investigate the effect of α -MG on the expression levels of transforming growth factor (TGF)- β 1 and matrix metalloproteinase-3 (MMP3) in hepatic stellate cells (HSCs) induced by TGF- β .

Methods: Immortalized HSCs and LX-2 cells were incubated with TGF- β with or without α -MG (5 and 10 μ M). The viability of LX-2 cells was assessed using the Trypan Blue Exclusion Method. The effect of α -MG on cell morphology and the mRNA expression levels of TGF- β 1, TGF- β receptor, and MMP3 was then evaluated.

Results: TGF- β enhanced the proliferation of HSCs and caused significant increases in the expression levels of TGF- β 1, TGF- β receptor, and MMP3. α -MG treatment reduced the proliferation of HSCs and decreased the expression levels of TGF- β 1, TGF- β 1 receptor, and MMP3.

Conclusion: α -MG is a potential antifibrotic agent due to its antiproliferative and antifibrogenic effects, mainly by suppressing the expression of TGF- β and MMP3 on the surfaces of activated HSCs.

Keywords: Alpha-mangostin, Transforming growth factor- β , Matrix metalloproteinase-3, Hepatic stellate cells.

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INTRODUCTION

Hepatic stellate cells (HSCs) are liver-specific mesenchymal cells that play a role in the pathogenesis of liver fibrosis [1,2]. HSCs are located in the perisinusoidal space and contain lipid droplets rich in Vitamin A. In the normal liver, stellate cells remain quiescent and play a role in maintaining the basal balance of the matrix membrane [2,3]. In response to liver injury, these cells receive signals from a wide variety of growth factors, cytokines, lipid mediators, and adipokines to promote survival at the sites of liver injury containing damaged hepatocytes and immune cells. HSCs then transdifferentiate to activated myofibroblast-like cells [2].

Transforming growth factor- β (TGF- β) is a key profibrogenic mediator that plays a major role in the activation of HSCs. Activated HSCs then secrete TGF- β in autocrine- and paracrine-dependent manners to mediate the production, degradation, and accumulation of molecules in the extracellular matrix (ECM) [1,4–6]. In humans, TGF- β exists in three homologous isoforms: TGF- β 1, TGF- β 2, and TGF- β 3. On binding to TGF- β receptor type II, TGF- β consecutively recruits TGF- β receptor type I to the complex and initiates TGF- β signaling, which causes the activation of SMAD-dependent and SMAD-independent pathways [7,8].

Activated HSCs express many ECM-associated proteins, including collagen type I, α -smooth muscle actin, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) [4,9]. Accumulation of these proteins by the ECM without sufficient degradation will form deposits that destroy and distort the normal hepatic structure, which results in cirrhosis and liver failure [1,2,6,8,10]. ECM degradation is mediated by MMPs, a family of zinc-dependent enzymes grouped into collagenases, gelatinases, stromelysins, and membrane-type MMPs. TIMPs regulate the MMP activity by binding to MMPs, thereby inhibiting proteolytic activities [11].

Although the TGF- β pathway is important in the pathogenesis of liver fibrosis, few targeted drugs have been developed. Pirfenidone, a pyridine derivate, is the only drug currently available to specifically block TGF- β 1 production for the long-term improvement of liver inflammation and fibrosis [9]. In addition, sorafenib is the only drug approved for the treatment of advanced-stage hepatocellular carcinoma. Hence, the development of new drugs and therapeutic regimens is an immediate medical necessity [12].

Various herbal products have been shown anti-liver fibrogenic activities by blocking TGF- β signaling and HSC activation [13]. For example, alpha-mangostin (α -MG), a xanthone derivate, is a major bioactive compound found in the tropical fruit *Garcinia mangostana* Linn. that has antifibrotic and antiproliferative activities, and has been shown to decrease TGF- β levels. However, the mechanism of action of this compound remains poorly understood. Therefore, the aim of the present study was to evaluate the effect of α -MG on the expression of TGF- β 1 and its receptors, and MMP-3 in HSCs induced by TGF- β [3,14].

METHODS

Cell culture

Immortalized human HSCs and LX-2 cells were cultured as described elsewhere [3].

Cell treatments

Cells were treated with one of four regimens in 10-cm culture dishes: (1) Medium only; (2) TGF- β at 2 ng/mL for 24 h, then new medium with TGF- β at 2 ng/mL for 24 h; (3) TGF- β at 2 ng/mL for 24 h, then new medium with TGF- β at 2 ng/mL and α -MG at 5 μ M for 24 h; or (4) TGF- β at 2 ng/mL for 24 h, then new medium with TGF- β at 2 ng/mL and α -MG at 10 μ M for 24 h. Afterward, the cells were harvested using

trypsin+ethylenediaminetetraacetic acid and subjected to cell viability analysis and RNA isolation. All experiments were performed 3 times in duplicate.

Cell viability analysis

Cell viability was assessed using the trypan blue exclusion assay and presented as the percentage of proliferating cells.

Quantitative reverse transcriptase-polymerase chain reaction

RNA was isolated from cells using the High Pure RNA Isolation Kit (Roche Applied Science, Penzberg, Germany) and further synthesized to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). mRNA analyses of TGF- β , TGF- β receptor, and MMP3 were performed using a LightCycler[®] 480 Instrument (Roche Applied Science) with FastStart Essential DNA Green Master mix (Roche Life Sciences) in accordance with the manufacturer's protocol. The number of quantification cycles (Cq) was calculated automatically using the machine's software. Cq data were processed using the Livak method to determine the expression levels. Beta-actin was used as a housekeeping gene.

The following primer sequences were used in this study: TGF- β (F): 5'-TGA ACC GGC CTT TCC TGC TTC TAC ATG-3'; TGF- β (R): 5'-GCG GAA GTC AAT GTA CAG CTG CCG C-3'; TGF- β 1 receptor (F): 5'-TTG CTG GAC CAG TGT GCT TCG-3'; TGF- β 1 receptor (R): 5'-CCA TCT GTT TGG GAT ATT TGG CC-3'; MMP3 (F): 5'-CAA AAC ATA TTT CTT TGT AGA GGA CAA-3'; MMP3 (R): 5'-TTC AGC TAT TTG CTT GGG AA-3'; β -actin (F): 5'-GCT GGA AGG TGG ACA GCG A-3'; and β -actin (R): 5'-GGC ATC GTG ATG GAC TCC G-3'.

Statistical analysis

The results are presented as the mean \pm standard error of the mean. One-way analysis of variance followed by the Tukey test was used for multiple comparisons. A probability $p > 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0d software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

There was no change in the morphology of LX-2 cells after 48 h of treatment with TGF- β with or without α -MG as observed under an inverted microscope at 10 \times magnification (Fig. 1).

The addition of TGF- β increased cell viability by 2-fold, as compared to untreated cells. Treatment with α -MG at the lower dose (5 μ M) failed to reverse cell viability, whereas the higher dose of α -MG reversed cell viability back to normal (Fig. 2).

The addition of TGF- β to the cell medium caused a significant increase in the mRNA expression levels of TGF- β 1 and its receptor. However, the increase in the mRNA expression of the TGF- β 1 receptor was not as great as that of TGF- β 1 (Fig. 3). The addition of α -MG decreased the mRNA expression of TGF- β 1 and strongly decreased that of the TGF- β receptor (Fig. 3).

The mRNA expression levels of MMP3 were significantly elevated by the treatment of TGF- β and reversed back to normal with α -MG at 10 μ M, but not at 5 μ M (Fig. 4).

DISCUSSION

HSCs are the main source of the ECM and important for the development of hepatic fibrosis. LX-2 cells are a human HSC line that is often used to study liver diseases. TGF- β treatment of LX-2 cells in this study was used as an *in vitro* model of hepatic fibrosis. TGF- β is a pleiotropic fibrogenic factor that is involved in a wide spectrum of cellular process, including differentiation, proliferation, apoptosis, and migration [6,15]. As is widely known, TGF- β can induce epithelial-mesenchymal transition (EMT) of cultured hepatocytes including HSCs. High levels of TGF- β as consequence of liver injury activate

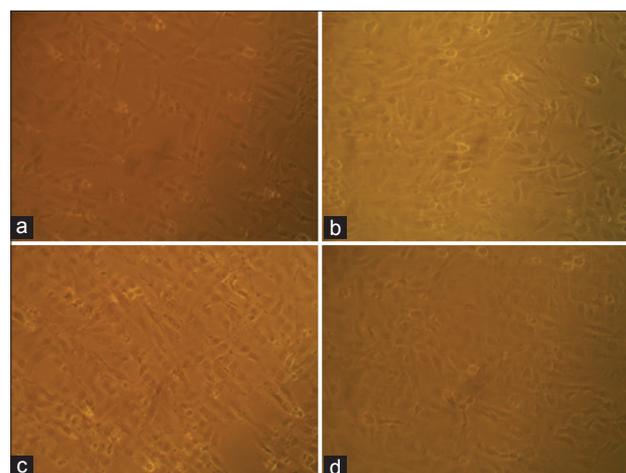


Fig. 1: Morphology of LX-2 cells after treatment with (a) medium only (untreated cells), (b) TGF- β at 2 ng/mL, (c) TGF- β at 2 ng/mL + α -MG at 5 μ M, or (d) TGF- β at 2 ng/mL+ α -MG at 10 μ M. Cells were observed under an inverted microscope at $\times 10$ magnification

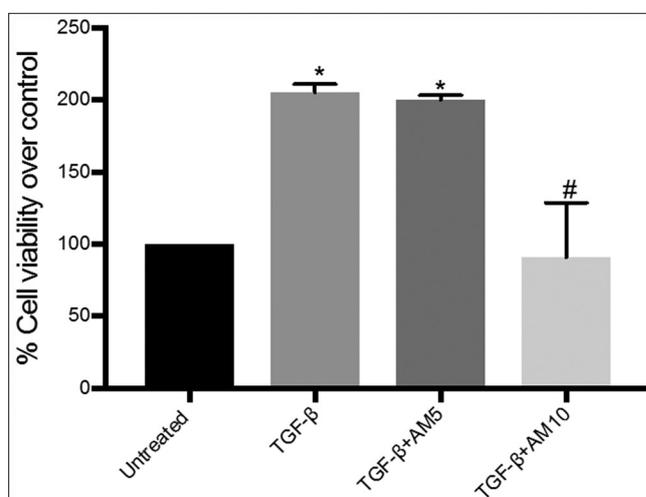


Fig. 2: Percentage of viable cells versus control cells after treatment with medium only (untreated cells), TGF- β (2 ng), TGF- β (2 ng)+ α -MG (5 μ M), or TGF- β (2 ng)+ α -MG (10 μ M). * $p < 0.05$ versus control (no treatment); # $p < 0.05$ versus TGF- β

HSCs and promote myofibroblast transdifferentiation as key effector cells in the fibrotic state [8,12,16–18]. Ho *et al.* used TGF- β at different concentrations to explore the short-term (3 days) effects on activated hepatocytes. In line with the results of the present study, they found no apparent morphological changes from a low treatment dose (0.2 ng/mL) for 3 days until 2 weeks or with a high dose (10 ng/mL) for 3 days. However, short-term treatment with TGF- β at 0.2 and 0.5 ng/mL upregulated the mRNA expression of EMT-related genes. In this study, there was no morphological differentiation between LX-2 cells after treatment with TGF- β with or without α -MG. This finding suggests that TGF- β treatment at 2 ng/mL for 24 h was not sufficient to induce morphological changes of HSCs [5]. However, further studies are needed to monitor EMT markers, such as vimentin and E-cadherin, to conclude that short-term (24 h) treatment with TGF- β at 2 ng/mL failed to induce EMT.

In contrast to the present study, Park *et al.* reported the transition of HSCs to spindle-like mesenchymal cells after treatment with TGF- β 1 for 12 h and these changes became even more evident after 48 h.

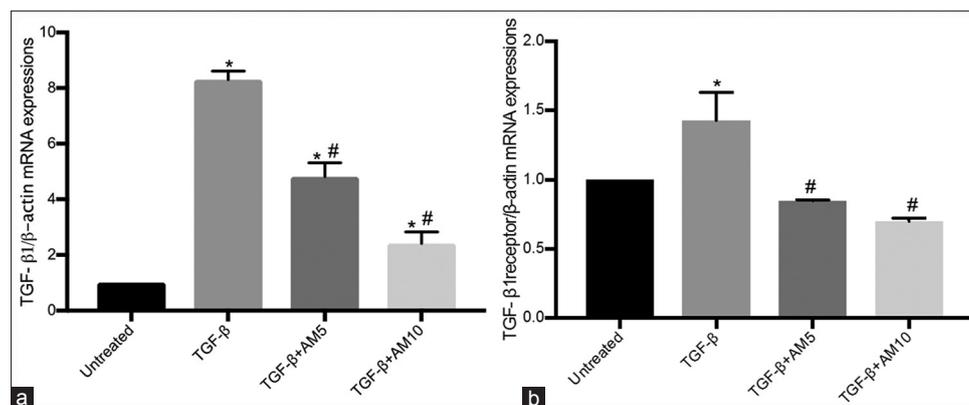


Fig. 3: mRNA expression levels of (a) TGF- β / β -actin and (b) TGF- β receptor/ β -actin after treatment of the cells with medium only (untreated cells), TGF- β at 2 ng, TGF- β at 2 ng+ α -MG at 5 μ M, or TGF- β at 2 ng+ α -MG at 10 μ M. * p <0.05 versus control (no treatment); # p <0.05 versus TGF- β

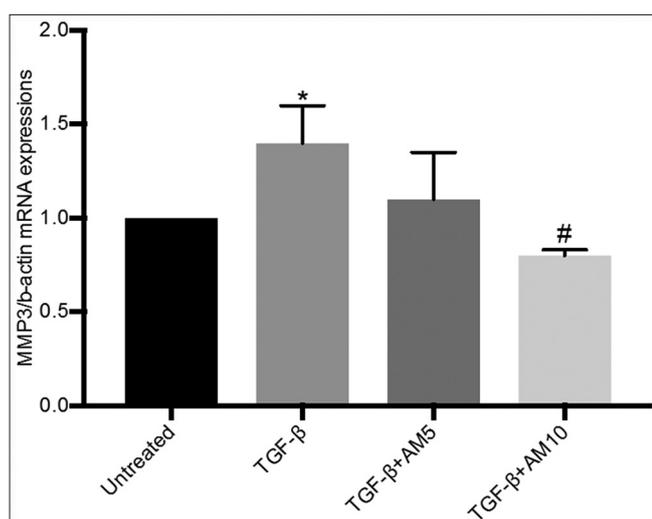


Fig. 4: Matrix metalloproteinase-3/ β -actin mRNA expression levels after cells were treated with medium only (untreated cells), TGF- β at 2 ng, TGF- β at 2 ng+ α -MG at 5 μ M, or TGF- β at 2 ng+ α -MG at 10 μ M. * p <0.05 versus control (no treatment); # p <0.05 versus TGF- β

This finding was supported by the downregulation of E-cadherin and upregulation of vimentin, as markers of the EMT process [8,19,20].

The results of the present study demonstrated that TGF- β treatment increased the number of viable cells by 2-fold as compared to untreated control cells. This increase in proliferation may due to TGF- β /SMAD-independent signaling, as the PI3K/Akt pathway can enhance Akt phosphorylation and increase HSC proliferation. A study by Zang *et al.* showed that TGF- β treatment increased the expression level of the proliferation marker Ki-67 in rabbit cornea cells, and Kang *et al.* found that TGF- β 1 treatment at 2 ng/mL at 24 and 48 h enhanced the HSC proliferation, whereas α -MG at 10 μ M suppressed this proliferation back to normal, which suggested the inhibitory activity of this substance against HSC proliferation [3,6].

In the healthy liver, high TGF- β 1 expression is only found in endothelial sinusoidal and Kupffer cells. On activation, HSCs modify the transcription of some key genes including α -smooth muscle actin, type 1 collagen, MMP-2, TGF- β 1, TGF- β receptor, and TIMP-1 and TIMP-2. Recent studies have found that the expression levels of signal transducer and activator of transcription 3 (Stat3) and TGF- β were increased in patients with advanced fibrosis [13,20]. Kim *et al.* reported an increase in the relative expression of TGF- β 1 after treatment with TGF- β 1 (2 ng/mL) for 12 h

in HSC-T6 cells, an immortalized rat HSC cell line [21]. These findings explain the increased expression of TGF- β 1 and TGF- β receptors after treatment with TGF- β .

As reported previously, TGF- β 1 activates HSCs and the activated HSCs synthesize and secrete ECM-degrading enzymes, known as MMPs, and their inhibitors, TIMPs. In the early phase, HSCs produce MMPs, which degrade the normal ECM in the liver. Afterward, fully activated HSCs suppress the expression of MMPs and promote that of TIMPs as natural inhibitors of MMPs [2,19]. The expression levels of MMP3 and TIMP-1 are upregulated during liver fibrosis. Kang *et al.* found that TGF- β 1 stimulation decreased MMP-2 expression in human prostate carcinoma cells, whereas the addition of α -MG decreased the expression levels of MMP2, MMP9, and urokinase plasminogen activator [6,22].

CONCLUSION

TGF- β acts as a gateway in intracellular signaling. Thus, there is a need to develop drugs to inhibit the intracellular activity of TGF- β . The results of this study confirmed that α -MG not only inhibited the proliferation of HSCs but was also an effective marker of fibrogenesis through the TGF- β pathway. Therefore, α -MG should be further investigated as a potential target for the treatment of liver fibrosis.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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