Simvastatin suppresses the proangiogenic microenvironment of human hepatic stellate cells via the Kruppel-like factor 2 pathway

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ABSTRACT

Background and aims: Statins are reported to have a beneficial effect on portal hypertension (PTH); however, the exact mechanism remains unknown. Hepatic stellate cells (HSCs) can be activated by transforming growth factor beta (TGFβ) and play an important role in angiogenesis leading to PTH. Statins potently stimulate the transcription factor, Kruppel-like factor 2 (KLF2), which can negatively regulate angiogenesis. Our present study aimed to investigate the anti-angiogenic potential of statins in HSCs through the KLF2 pathway.

Method: TGFβ-induced human HSCs were exposed to simvastatin. Cell viability and proliferation were determined by MTT and BrdU-proliferation assays, respectively. Cell migration was investigated using a transwell and wound-healing assays. Gene quantification was measured by real-time polymerase chain reaction. Protein expression was detected by western blot analysis and immunohistochemistry. Inflammatory factors were measured using enzyme-linked immunosorbent assays.

Result: Simvastatin was found to reduced cell migration and proliferation and inhibit expression of alpha smooth muscle actin in TGFβ-induced HSCs. Furthermore, simvastatin promoted already increased mRNA and protein levels of KLF2 in TGFβ-induced HSCs. In accordance with KLF2 overexpression, simvastatin increased production of endothelial nitric oxide synthesis (eNOS) and downregulated expression of some proangiogenic proteins, such as vascular endothelial growth factor, hypoxia inducible factor-1α and nuclear factor-kappa B in TGFβ-induced HSCs. At the same time, secretion of interferon-gamma increased in TGFβ induced HSCs, which was decreased by simultaneous addition of simvastatin.

Conclusion: Simvastatin suppressed the proangiogenic environment of HSCs activated by TGFβ, and KLF2 pathway is involved in the course.

Key words: Simvastatin. Angiogenesis. Hepatic stellate cell. Kruppel-like factor 2.

INTRODUCTION

Portal hypertension (PTH) is one of the most common complications caused by cirrhosis, which commonly leads to variceal hemorrhage and bleeding-related death. The most critical reason for induced PTH is increased intrahepatic vascular resistance (1). Fibrosis and intrahepatic vasoconstriction are classical mechanisms that account for increased intrahepatic vascular resistance in PTH (2). Recently, research has focused on the relationship of intrahepatic angiogenesis and PTH (3,4).

In the development of PTH, activated hepatic stellate cells (HSCs) are responsible for all pathological changes in the cirrhotic liver. Tissue and serum levels of active TGFβ are elevated in fibrosis, and application of exogenous TGFβ can induce liver fibrosis (5). Although previous investigations of HSCs in the liver have focused on their unique capacity to deposit collagen matrix, ensuing a wound-healing response, the contribution of HSCs to hepatic microvascular structure has only recently become...
recognized (6). In response to liver damage, HSCs are activated and largely proliferate and migrate to the surrounding sinusoidal endothelial cells (SECs), wrapping around the vascular tubes that have lost their normal phenotype. HSCs may also produce angiogenic molecules, such as vascular endothelial growth factor (VEGF), and contribute to angiogenesis through direct or indirect mechanisms (7). Because of their major role in inducing PTH, HSCs are excellent targets for therapy.

Statins are widely used in the clinic for their cholesterol-lowering properties. However, several clinical trials have shown that statins also have cholesterol-independent benefits, including the improvement of liver fibrosis and PTH (8,9). Researchers have demonstrated that statins can inhibit activation of HSCs (10-12); however, the actual mechanism of action for statin therapy on the proangiogenic environment of HSCs and relevant signal pathway remains yet to be elucidated.

Some reports have shown that statins can strongly induce the vasoprotective transcription factor, Kruppel-like factor 2 (KLF2), in the vascular system (13-16). This in turn can protect the endothelial cell phenotype against inflammation and negatively regulate angiogenesis (17,18). We therefore hypothesize that statins may suppress the proangiogenic microenvironment of HSCs through the KLF2 pathway. The aim of this study was to investigate the effect of statins on the activation of human HSCs and their proangiogenic microenvironment.

METHODS

Cells and cell culture

LX-2 cells (kindly provided by Dr. Guo), a low passage cell line, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 % heat-inactivated fetal bovine serum (FBS). Cells were subcultured two or three times a week by trypsinization in a 0.25 % activated fetal bovine serum (FBS). Cells were subcultured medium (DMEM) supplemented with 5 % heat-inactivated and largely proliferate and migrate to the surrounding sinusoidal endothelial cells (SECs), wrapping around the vascular tubes that have lost their normal phenotype. HSCs may also produce angiogenic molecules, such as vascular endothelial growth factor (VEGF), and contribute to angiogenesis through direct or indirect mechanisms (7). Because of their major role in inducing PTH, HSCs are excellent targets for therapy.

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MTT assay

Cell viability was determined using the MTT assay. This method is based on the capability of living cells to reduce light colored tetrazolium salts into an intense color formazan derivation. This reduction process requires functional mitochondria, which become inactive within a few minutes after cell death. HSC cells (2×10^5) were seeded in a 96-well culture plate for 24 h and then starved for 24 h in serum-free DMEM. Medium was then replaced with DMEM containing 5 % FBS with 10 ng/ml TGFβ, or in the presence of simvastatin. HSCs were then incubated for 4 h with MTT solution (5 mg/ml), and light absorbance was detected at a wavelength of 570 nm. The results were plotted as means ± standard deviations (SD) of two independent experiments, having three determinations per sample for each experiment.

BrdU-proliferation assay

The effect of simvastatin on DNA synthesis of activated HSC cells was investigated by performing BrdU enzyme-linked immunoabsorbent assay (ELISA). Briefly, 2×10^3 cells were seeded in 96-well plates. Subconfluent cell cultures were treated with 10 ng/ml TGFβ alone or 10 ng/ml TGFβ combined with simvastatin for 24 h. During the 24 h experimental time point, cells were labeled with 10 µM BrdU and incorporation was measured according to the manufacturer’s instructions (Roche). This experiment was conducted three times.

Wound-healing assay

Cell migration was studied using an in vitro wound healing assay. HSC cells (3×10^5) were seeded onto 4-cm culture dishes (Nunc) in 5 % FBS-DMEM for 24 h. One linear wound was then scraped in each well with a sterile pipette tip (p300). All cellular debris were removed and medium was replaced with fresh 0.5 % FBS-DMEM alone or together with simvastatin and 10 ng/ml TGFβ. Control cells were cultured in 0.5 % FBS-DMEM. The number of cells that had migrated into the wounded area after 24 h was estimated in the control and in the cultures treated with TGFβ and simvastatin. Migrated cells were observed on micrographs, and the results were shown as the mean number of cells from 100 randomly chosen wounded areas. This experiment was conducted three times.

Transwell migration assay

To mimic the HSC perisinusoidal (Disse) space microenvironment in vivo, polyvinyl/pyrrolidone-free polycarbonate membranes with 8 µm pores, which separate the upper and lower wells in a transwell chamber (Corning), were coated with type IV collagen on the upper side (50 µg/ml) and type I collagen on the lower side (50 µg/ml). The bottom wells of the chamber were filled with DMEM, and 2×10^4 of serum-starved LX-2 cells/well, were added into the upper chambers. TGFβ or simvas-
tatin were added into the upper chambers as direct haptotactic stimulants, and into the lower chamber as indirect chemotactic stimulants, to mimic the in vivo autocrine and paracrine mechanisms of cytokines, respectively. The transwell chamber was incubated at 37 °C for 24 h to allow migration of cells through the membrane into the lower chamber. The migrated cells were stained with May-Grunwald-Giemsa and counted in six random fields of view on a phase contrast microscope. This experiment was conducted three times and each time was assessed in three fields.

**Cytokine ELISAs**

HSCs were cultured for 72 h and serum-starved for 24 h prior to assaying. HSC supernatants were collected for analysis of interferon-gamma (INFγ), interleukin (IL)-2, IL-4 and IL-5 with ELISA kits, following the manufacturer’s protocol (eBioscience). All experiments were performed for 3 times.

**Immunohistochemistry study**

LX-2 cells were stimulated for 24 h with TGFβ in the presence or absence of simvastatin. Cells were fixed in phosphate-buffered saline containing 4 % paraformaldehyde at room temperature for 30 minutes and were penetrated in blocking solution containing 0.3 % Triton X-100, and incubated overnight at 4 °C with either anti-alpha smooth muscle actin (anti-αSMA, 1:100, Sigma-Aldrich) or anti-KLF2 (1:100, Abcam) in 1 % BSA solution. Cells were then incubated with secondary antibodies for 1 h at 37 °C. After incubation, cell staining was observed in 10 fields of view at ×40 magnification. This experiment was conducted twice.

**Western blotting analysis**

HSC cells (1x10⁴) were plated in six-well plates for 48 h and then harvested. Total protein was extracted, separated on 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocking with 5 % non-fat dry milk in Tris-buffer solution, membranes were probed with primary monoclonal antibodies specific to αSMA (1:1000; Sigma Aldrich), KLF2 (1:1000, Abcam), endothelial nitric oxide synthesis (eNOS), nuclear factor-kappa B (NF-kappaB; 1:1000, Santa Cruz), hypoxia inducible factor (HIF)-1a, and HIF-2a (1:1000, Sigma Aldrich). Horseradish peroxidase-conjugated secondary antibodies (Beyotime) were then applied, and blots were developed using the western blotting detection system.

**Real-time polymerase chain reaction (PCR)**

RNA was isolated from LX-2 cells using a Nucleospin RNAII kit (Takara) followed by reverse transcription with M-MLV reverse transcriptase according to the manufacturer’s instructions (Takara). The primers used for KLF2 were as follows: Forward: AAGACCTACACCAAGAGTTCG; Reverse: CTACATGTGCCGTTTTCATGTG. Expression of β-actin was amplified as an internal standard. All experiments were performed by the same investigator with no knowledge of the corresponding grouped data.

**Statistical analysis**

Quantitative results were expressed as means ± SD. Statistical analysis was assessed by the Student-t test (between two groups) or Student-Newman-Keuls test (among three or more groups), with SPSS 16.0 software (SPSS, Chicago, IL). A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Role of simvastatin in TGFβ-induced proliferation and migration of HSCs**

We examined whether simvastatin could regulate the two principal indicators of HSC activation, proliferation and migration. TGFβ (10 ng/ml) was found to increase cell proliferation by 1.2-fold and migration by 3-fold compared with the control. Conversely, simvastatin (1 µM) significantly reduced HSC cell proliferation and migratory rates by about 50 % (Fig. 1). These data demonstrate the importance of simvastatin in TGFβ-mediated HSC proliferation and migration.

**Effect of simvastatin on αSMA expression in TGFβ-activated LX-2 cells**

αSMA is a major marker of HSC activation. We found that 10 ng/ml TGFβ promoted expression of αSMA twice that of the control, and 1 µM simvastatin suppressed upregulation by 1.5 fold of the control (Fig. 2A and B). Additionally, in LX-2 cells, αSMA was expressed in the cytoplasm according to immunohistochemistry (IHC) results (Fig. 2C).

**KLF2 expression in HSCs**

We found that simvastatin strongly promoted expression of KLF2 in HSCs. KLF2 mRNA levels increased approximately 1.5-fold in HSCs treated with 1 µM simvastatin, in accordance with increased protein expression (Fig. 3A). In-
duced by TGFβ, both mRNA and protein levels of KLF2 expression increased approximately 2-fold in HSCs, and interestingly, simultaneous addition of simvastatin caused expression to increase approximately 4-fold (Fig. 3 A and B).

Localization of KLF2 using IHC revealed that KLF2 was barely detected in control HSCs, confirming lower expression of this transcription factor in control HSCs. When induced by TGFβ and simvastatin, KLF2 protein expression was specifically detected in the plasma of LX-2 cells (Fig. 3C).

**KLF2-target protein expression**

Analysis of KLF2-targeted genes and proteins involved in vascular physiology showed that, in the TGFβ group, eNOS protein decreased compared with the control, while HIF-1α, NF-κB and VEGF showed no difference in TGFβ group. Interestingly, when simvastatin was added simultaneously, compared with the TGFβ group, eNOS expression increased while HIF-1α, NF-κB and VEGF decreased to different extents. Ang-2 showed no difference in 3 groups (Fig. 4A).

**Inflammatory factor secretion**

After treatment with TGFβ or TGFβ combined with simvastatin, HSC supernatants were harvested for ELISA detection. Our experiments demonstrated that INFγ was increased in TGFβ-induced HSC supernatants, but secretion was reduced when co-treated with simvasta-
tin (Fig. 4B). There was no significant difference in production of IL-2, IL-4 and IL-5 between the three groups (data not shown).

DISCUSSION

PTH is initiated predominantly through intrahepatic vascular resistance. Recent data suggest that intrahepatic angiogenesis may also be involved in sinusoidal resistance and PTH (2,19). Angiogenesis is characterized by increased mural cell coverage of vessels by activated HSCs (1).

Recent advances suggest that HSCs are major contributors to angiogenesis (20). Activated HSCs proliferate, migrate and recruit to the surroundings of vessel lumen, thus providing durability to the vessels in a direct way. Conversely, indirect mechanisms are also likely to be important and include the ability of HSCs to secrete angiogenic molecules, such as VEGF and Ang-2 (1,21). Additionally, activated HSCs release inflammatory mediators, which can elicit an angiogenic response through the induction of HIF-1α (22,23) and NF-κB transcriptional activity (2).

Statins are considered ideal drugs for PTH as they reduce portal pressure by decreasing intra-hepatic vascular resistance (8,24-26). Some groups have previously investigated the effect of statins on hepatic inflammation and fibrosis (12). However, studies into the cellular mechanism of this effect are limited. Trebicka has shown that statins can decrease HSC contraction by a nitric oxide-independent mechanism (10). Other studies have demonstrated that statins can suppress HSC apoptosis (27); however, the exact effect of statins on the proangiogenic microenvironment of HSC remains unclear. We aimed to investigate the effect of statins on activation of HSCs and the
proangiogenic microenvironment. As TGFβ is the most potent cytokine known to activate HSCs and angiogenesis (28), this study is focused on the specific role of statins in TGFβ-induced HSCs.

Our study explored the multiple effects of the statin simvastatin, on TGFβ-activated HSCs. Treatment of HSCs with simvastatin influenced several parameters of TGFβ-induced HSCs activation, including a decrease in αSMA production and inhibition of HSC proliferation and migration. Previous research shows that statins can reduce activation of HSCs in vivo (10,29), and our results support that statins can also suppress TGFβ-induced HSCs activation in vitro.

A large number of studies have shown that KLF2, a vasoprotective transcriptional factor, is endothelial-specific and its expression confers to endothelial protection against inflammation and negative regulation of angiogenesis (30-32). KLF2 can also modulate blood vessel maturation through smooth muscle cell migration (33). Previous studies have shown that simvastatin can potently induce KLF2 in the vascular system (13,14,34,35), which may protect the normal endothelial phenotype and inhibit the angiogenic process. Studies have also shown that KLF2-overexpression happens in the vasculature of cirrhotic patients (36), in activated HSCs (37)

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**Fig. 3.** A. Expression of KLF2 protein in HSCs determined by immunoblot. Fold changes between the control and representative western blots are shown. B. KLF2 gene mRNA expression level in HSC with different treatments. Values for KLF2 amplification from cell cDNA were normalized to an endogeneous reference gene. Fold changes compared with the control are shown. All data are shown as means ± SD of triplicates, and were measured twice. C. KLF2 protein expression in HSCs in different condition. Representative images were visualized with a light microscope and are shown at x40 magnification. The experiment was repeated three times. *p < 0.05 vs. control; #p < 0.05 vs. TGFβ group; p < 0.05 vs. control; #p < 0.05 vs. TGFβ group.
and in cirrhotic SECs (35). Marrone found that statins can promote angioprotective factors in SECs through the KLF2 pathway (16). Our study reports for the first time, that simvastatin upregulated protein expression of KLF2 in human HSCs, and that KLF2 was expressed both in the cytoplasm and nucleus. Interestingly, overexpression of KLF2 protein increased following TGFβ treatment, and the simultaneous addition of simvastatin made the increase more significant. This result suggests that the abundance of KLF2 protein may have a beneficial effect on the antiangiogenic microenvironment of completely activated HSCs.

We also detected KLF2 target gene expression. Expression of KLF2 is reported to stimulate eNOS expression, which increases the bioavailability of vasodilators and reduces intrahepatic resistance (38–41). KLF2 can also potently suppress HIF-1α expression (42–44), which downregulates proangiogenic factors, such as VEGF and Ang-2. HIF-1α, conversely, promotes angiogenesis by stimulating the NF-κB-dependent inflammatory pathway (45,46). The present study investigated for the first time, the expression of angiogenesis-relevant proteins in HSCs. Our findings demonstrated that, TGFβ-induced HSCs expressed high level protein NF-κB, HIF-1α and VEGF, but expression of eNOS decreased. Interestingly, simultaneous addition of simvastatin suppressed the protein change caused by TGFβ. Our results suggest that KLF2 overexpression induced by simvastatin can suppress the increase of proangiogenic factors such as VEGF, NF-κB and HIF-1α, stimulated by TGFβ in HSCs. Moreover, simvastatin-induced KLF2 can promote production of eNOS, a vasodilative factor, which decreased in TGFβ-activated HSCs. Therefore, simvastatin inhibited the proangiogenic microenvironment of HSC by modulating KLF2 expression and its downstream gene cascade.

Our study also highlighted some interesting inflammatory mechanisms by which simvastatin leads to the suppression of TGFβ-induced activation in HSCs. For example, TGFβ stimulation also led to the upregulation of IFNγ, an angiogenic cytokine and growth factor, while simvastatin inhibited upregulation of IFNγ. Some reports have demonstrated that IFNγ is involved in angiogenesis (47–49), but the exact mechanism remains unclear.

In summary, our data revealed that simvastatin inhibited TGFβ-induced migration, proliferation and activation of HSCs, which might be a key mechanism for its inhibitory action on the direct proangiogenic interaction between HSCs and SECs. Additionally, simvastatin exerted anti-angiogenic function, which is associated with the expression of KLF2 and its downstream genes and proteins.
ACKNOWLEDGMENTS

We thank Mei-Yu Hu and Jian-Jun Jin from the Biomedical Research Center for their technical support and thank the Biomedical Research Center of Zhongshan Hospital for experimental equipment supply.

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