의학석사 학위논문

Bile acid-mediated induction of hepatic stellate cell activation and invasion via Mcl-1 and COX-2 in hypoxic conditions

저산소조건에서 담즙산이 Mcl-1 및 COX-2 매개 간성상세포의 활성화에 미치는 영향

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서울대학교 대학원
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Bile acid-mediated induction of hepatic stellate cell activation and invasion via Mcl-1 and COX-2 in hypoxic conditions

by

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Abstract

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Background: Activated hepatic stellate cells (HSCs) are the major subtype of stromal cells in the liver tumor microenvironment which can promote the growth and migration of hepatocellular carcinoma (HCC) cells. Indeed, senescent and cancer-associated fibroblasts express numerous inflammatory and tumor promoting factors that are collectively referred to as the senescence-associated secretory phenotype (SASP). In the present study, we investigated the mechanisms of bile acid-mediated induction of HSC activation as the SASP in hypoxic conditions.

Methods: The immortalized human stellate cells (LX-2 cells) were used in this study. Invasion assay were done to evaluate the invasion of HSCs either in a normoxic or hypoxic conditions. IL-8 mRNA was quantitated using real-time PCR. To investigate the mechanisms, western blot analyses were performed either in a normoxic or hypoxic conditions.

Results: Bile acid significantly increased the invasion of HSCs in hypoxic conditions as compared to that in normoxic conditions by IL-8 expression, which is an inflammatory cytokine involved in tumor promotion and key component of the SASP. Bile acid increased protein expressions of the mesechymal markers including α-SMA and vimentin in HSCs. Moreover, bile acid increased the protein expressions of Mcl-1 and cyclooxygenase-2 (COX-2) in HSCs. The inhibitors of either Mcl-1 induction by siRNA transfection or COX-2 activity by celecoxib decreased the bile acid-mediated HSC invasion in hypoxic conditions. Mcl-1 and COX-2 induction was found to be due to transcriptional enhancement dependent on TGR-5 activation.

Conclusions: Bile acid-mediated induction of HSC activation and invasion as the SASP was due to TGR-5 dependent overexpression of Mcl-1 and COX-2, which may lead to HCC progression in hypoxic conditions.

Keywords: hepatic stellate cell, bile acid, Cyclooxygenase-2, Mcl-1, hypoxia
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List of abbreviations and symbols

α-SMA	α-smooth muscle antibody
AP-1	AMP-activated protein kinase
Bcl-2	B-cell lymphoma 2
COX-2	cyclooxygenase-2
CXCL9	chemokine (C-X-C motif) ligand 9
DC	deoxycholate
DMEM	Dulbecco's modified Eagle's medium
GROα	growth related oncogene-α
GPBAR1	G protein-coupled bile acid receptor 1
HCC	hepatocellular carcinoma
HIF-1	hypoxia-inducible factor-1
HRE	hypoxia-responsive element
HSC	hepatic stellate cell
IL	interleukin
JNK	c-Jun-N terminal kinase
LX-2	immortalized human stellate cell
MAPK	mitogen-activated protein kinase
M-BAR	membrane-type receptor for bile acids

Mcl-1	myeloid cell leukemia 1
PGE2	prostaglandin E2
RT-PCR	reverse transcriptase polymerase chain reaction
SASP	senescence-associated secretory phenotype

Introduction

Human hepatic stellate cells (HSCs) comprise approximately 15% of all liver cells and play an important role in hepatic injury and fibrosis. Recently, these cells were postulated to form a component of the pro-metastatic liver microenvironment, which can promote the growth and migration of hepatocellular carcinoma (HCC) cells. Following liver injury, quiescent HSCs are activated and converted into highly proliferative myofibroblast-like activated HSCs or senescent HSCs that affect cancer cell proliferation and invasiveness in a paracrine manner [1-4]. Indeed, senescent and cancer-associated fibroblasts express numerous inflammatory and tumor promoting factors that are collectively referred to as the senescence-associated secretory phenotype (SASP) [4]. This SASP develops slowly over several days after genotoxic stress and induces an epithelial-mesenchymal transition and invasiveness, which are hallmarks of malignancy, due to a paracrine mechanism that depends largely on the SASP factors interleukin (IL)-6 and IL-8 [4].

In the previous studies, we demonstrated that bile acids induce cyclooxygenase-2 (COX-2) and Mcl-1 protein expression in HSCs and cholangiocytes, which leads to survive against bile acid-induced apoptosis [5-7] unlike hepatocytes undergoing apoptosis [8,9]. COX-2 is an enzyme that is involved in prostanoid metabolism and may promote

cellular growth, inhibit apoptosis, and support angiogenesis [10,11]. Moreover, there is a growing body of evidence that COX-2 expression is correlated to cancer invasion and metastasis by lowering the activity of E-cadherin, which inhibits the separation of cancer cells from tissues by matrix metalloproteinase upregulation [12]. Mcl-1 is a potent anti-apoptotic Bcl-2 family protein induced by a variety of stimuli. Specifically, it blocks cytochrome *c* release, which is required for the intrinsic apoptosis pathway [13]. During liver damage and secondary inflammatory reaction, hypoxia in a local micro-environment is inevitable, which could cause DNA damage of a significant magnitude to induce cellular senescence. Therefore, we hypothesized that bile acids along with hypoxia may induce SASP, which could lead to HSC activation and invasion.

In this study, we further investigated how quiescent HSCs are activated in hypoxic conditions with bile acid exposure using several in vitro assays. We specifically investigated the mechanisms of HSC activation via COX-2 and Mcl-1 protein expression as the SASP in hypoxic conditions. Thus, the mechanism of these signaling cascades may provide new targets for preventing the progression of HCC in hypoxic conditions by inhibiting HSC activation.

Materials and methods

Cell Cultures

The immortalized human stellate cells, LX-2 cells [14], were used in this study. LX-2 cells were cultured either under standard culture conditions (20% O2 and 5% CO2 at 37℃) or under hypoxic conditions (1% O2, 5% CO2, and 94% N2 at 37℃) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100,000 U/L penicillin, 100 mg/L streptomycin, and 100 nM insulin. Cells were serum-starved over 12 h before bile acid, deoxycholate (DC), treatment to avoid the confounding variable of serum-induced signaling. Medium was replenished at 12 and 24 h after plating and every 48 h thereafter. The purity of isolated HSCs was estimated through vitamin A autofluorescence. Experiments were conducted using cells with an activated or myofibroblast-like phenotype 10 days after plating.

Invasion assay

In vitro invasion assays were performed using 24-well chambers to study invasion of LX-2 cells either in a normoxic or hypoxic conditions. Inserts were transferred into wells and coated with Matrigel (BD Biosciences, Billerica, MA, USA) for 30 min at 37℃. LX-2 cells were

suspended in serum-free medium and implanted on the Matrigel- coated upper chambers (5 × 104 cells/chamber), and DMEM containing 10% fetal bovine serum was added to the lower chambers. The cells were incubated for 6 h at 37℃ and then either pretreated with 20 μM celecoxib or 10 μM TGR-5 inhibitor (MDL-12,330A) or left untreated. After 1 h of pretreatment, the cells were incubated in the presence or absence of DC (200μM) for 24 h and then stained with 4 μg/mL calcein AM (BD Biosciences, Billerica, MA, USA) in Hank's Balanced Salt Solution (HBSS) at 37℃ for 90 min. Invasion ability was determined as relative fluorescence units measured at an excitation of 494 nm and an emission of 517 nm using a multifunctional plate reader (EnVision Multilabel Reader; PerkinElmer Inc., Waltham, MA, USA).

Immunoblot assay

Cells were lysed for 20 min on ice with lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium DC, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, 1 mmol/L NaF, and 1 ug/mL each or aprotinin, leupeptin, and pepstatin) and centrifuged at 14,000 g for 10 min at 4℃. Samples were resolved by 7.5% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, blotted with appropriate primary antibodies at a dilution of 1:1000, and incubated

with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X-OMAT film (Kodak, New Haven, CT, USA). The primary antibodies used were: rabbit anti-Mcl-1, goat anti-COX-2, mouse anti-p21, and goat anti-actin from Santa Cruz Biotechonology Inc. (Santa Cruz, CA, USA), mouse anti-α-smooth muscle antibody (SMA), and mouse anti-vimentin from BioGenex Laboratories Inc. (San Ramon, CA, USA), and rabbit anti-CAP43 from Invitrogen Corporation (Camarillo, CA, USA).

Real-time PCR analysis

Total RNAs were extracted from the cells using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The cDNA template was prepared using oligo(dT) random primers and Moloney Murine Leukemia Virus reverse transcriptase [15]. After the reverse transcription reaction, the cDNA template was amplified by PCR using Taq polymerase (Invitrogen). IL-8 was quantitated using real-time PCR (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) using SYBR green as the fluorophore (Molecular Probes, Eugene, OR, USA). After electrophoresis in 1% agarose gel, the portion of gel containing the expected PCR product of IL-8 was excised, and the product was eluted

into Tris-HCl using a DNA elution kit (Gel extraction kit; Qiagen, Vanlencia, CA, USA). The eluted and purified PCR product was quantitated using a spectrophotometer (Beckman DU 7400) at 260 nm. An inverse linear relationship was obtained between cycle number and copy number, and the resulting standard curve was used to calculate the copy numbers/mL in experimental samples. Results were expressed as ratios of IL-8 copies/mL in samples stimulated with DC to those in control samples.

Materials and reagents

A specific double-stranded 21-nucleotide RNA sequence homologous to the target message was used to silence Mcl-1 in LX-2 cells [16]. Cells grown to subconfluence for 3-7 days were transfected with 40 nM siRNA for human Mcl-1 (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The COX-2 inhibitor, purified celecoxib, was from Pharmacia Korea (Seoul, Korea). The TGR-5 inhibitor, MDL-12,330A, was from Sigma-Aldrich Co. (St. Louis, MO, USA).

Statistical analysis

Differences between two groups were analyzed using the two-tailed Student's t tests or the Mann-Whitney *U* tests. All statistical analysis

was performed using PASW version 21.0 (IBM, Chicago, IL, USA). Statistical significance was defined as a P value less than 0.05.

Results

Bile acid changed the protein expressions of the senescent markers in HSCs

We first investigated whether or not the transcript levels of the senescent markers were changed by the DC treatment in LX-2 cells to further elucidate how the SASP contributes to the activation and invasion of HSCs. As shown in Fig. 1, the treatment of LX-2 cells with DC in hypoxic conditions resulted in the increased protein expressions of p21, a potent cyclin-dependent kinase inhibitor that functions as a regulator of cell cycle progression at the G1 and the S phase and mediates cellular senescence [17]. Thus, it could be inferred that cellular senescence is induced in response to the exposure of both bile acid and hypoxia. CAP43, also called Drg1/RTP/Rit42/NDRG1, has been shown to be upregulated by nickel compounds and hypoxic stress and may be involved in HCC progression [18,19]; however, the protein expression of CAP43 did not definitively increase in the presence of bile acid in normoxic or hypoxic conditions.

Bile acid significantly increased the invasion of HSCs by IL-8

We next examined whether or not bile acid treatment increases the invasion of HSCs in normoxic or hypoxic conditions, and if so, how the SASP contributes to the invasion of HSCs. The invasion of HSCs

significantly increased more in hypoxic conditions than in normoxic conditions (Fig. 2A). The treatment of LX-2 cells with DC in both normoxic and hypoxic conditions significantly increased the invasion of HSCs (Fig. 2B). The levels of IL-8 determined by real-time PCR analysis were promptly increased when LX-2 cells were cultured with DC in hypoxic conditions. Moreover, these increased levels of IL-8 were more prominent with DC treatment than without DC treatment (Fig. 2C). These findings indicate that bile acid significantly increases the invasion of HSCs by IL-8, which is one of the most important SASP factors.

Bile acid increased the protein expressions of Mcl-1 and COX-2 in HSCs

In our previous study, we illustrated the induction of Mcl-1 and COX-2 in HSCs by bile acid [5]. In this study, we sought to investigate the bile acid-mediated induction of Mcl-1 and COX-2 in HSCs under hypoxic conditions as well as under normoxic conditions. The treatment of LX-2 cells with DC increased Mcl-1 protein expression levels in both normoxic and hypoxic conditions. In addition, the degree of Mcl-1 protein expression under hypoxic conditions was relatively greater than those under normoxic conditions (Fig. 3A). The bile acid-induced induction of COX-2 was also observed; however, it was much

more remarkable under hypoxic conditions than under normoxic conditions (Fig. 3B). These findings showed that bile acid increases Mcl-1 and COX-2 protein expression levels in HSCs, most notably under hypoxic conditions.

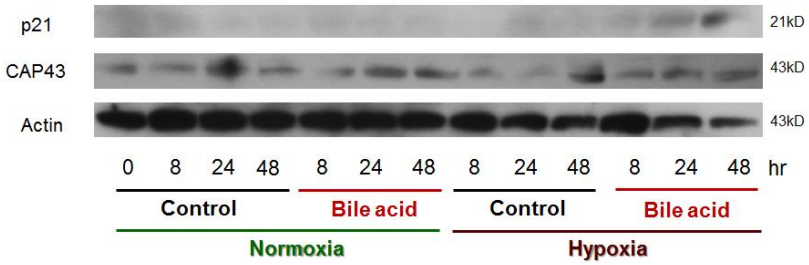

Figure 1. Bile acid changed the protein expressions of the senescent markers in HSCs. LX-2 cells were cultured in the presence of media (control) or DC (200 μM) for the indicated times either under normoxic or hypoxic conditions. At each time point, cells were lysed and immunoblot analysis was performed for p21 and CAP43. Immunoblot for actin was performed as a protein loading control.

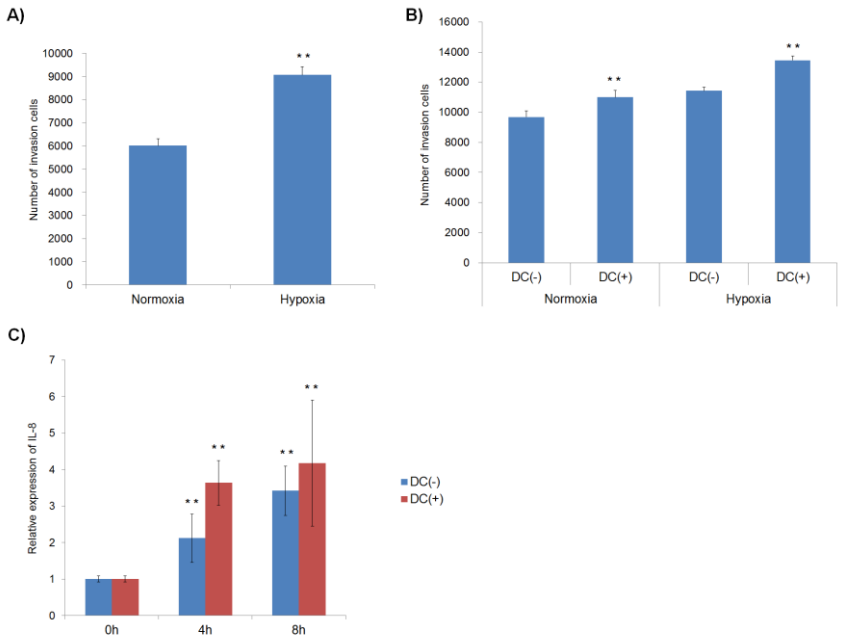

Figure 2. Bile-acid significantly increased the invasion of HSCs by IL-8. (A) LX-2 cells were cultured either under standard culture conditions (20% O2 and 5% CO2 at 37℃) or under hypoxic conditions (1% O2, 5% CO2, and 94% N2 at 37℃). (B) LX-2 cells were cultured in the presence of media (control) or DC (200 μM) for the indicated times either under normoxic or hypoxic conditions. Invasion ability was determined by invasion assay. (C) LX-2 cells were cultured in the presence of media (control) or DC (200 μM) for the indicated times. IL-8 was quantitated using real-time PCR using SYBR green as fulorophore and a LightCycler. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

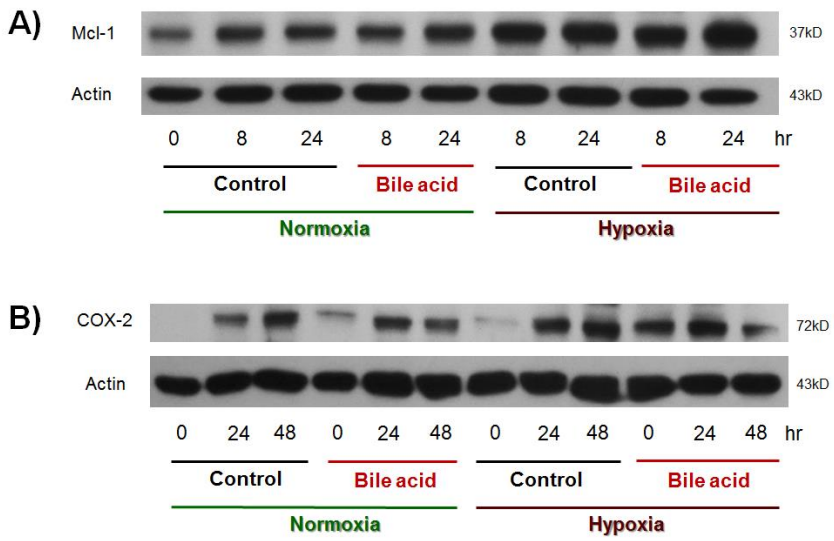

Figure 3. Bile acid increased the protein expressions of Mcl-1 and COX-2 in HSCs. LX-2 cells were treated with media (control) or DC (200 μM) either under normoxic or hypoxic conditions. (A) At each time point, cells were lysed and immunoblot analysis was performed for Mcl-1 and actin. (B) Similarly, at each time point, cells were lysed and immunoblot analysis was performed for COX-2 and actin.

The selective inhibition of Mcl-1 and COX-2 and HSC invasion under hypoxic conditions

To selectively inhibit Mcl-1 induction, LX-2 cells were transfected with Mcl-1-specific siRNA. While the treatment of LX-2 cells with DC in the control group significantly increased HSC invasion, Mcl-1 siRNA transfected LX-2 cells with DC treatment showed an attenuated degree of HSC invasion in hypoxic conditions (Fig. 4A). Similarly, COX-2 inhibition by celecoxib resulted in the attenuation of bile acid-mediated HSC invasion in hypoxic conditions, while a remarkable degree of increased invasion was observed in cells not treated with celecoxib (Fig. 4B). Thus, we found that the inhibitors of either Mcl-1 (by siRNA transfection) or COX-2 (by celecoxib) attenuate the bile acid-mediated HSC invasion in hypoxic conditions.

The selective inhibition of TGR-5 suppressed the bile acid-mediated HSC invasion and the protein expressions of Mcl-1 and COX-2 under hypoxic conditions

To further elucidate the pathway regulating the bile acid inductions of these proteins, Mcl-1 and COX-2, LX-2 cells were treated with MDL-12,330A, a TGR-5 inhibitor. TGR5, also known as G protein-coupled bile acid receptor 1 (GPBAR1) or membrane-type receptor for bile acids (M-BAR), functions as a cell surface receptor for bile acids, which

induces the production of intracellular cAMP and the activation of a mitogen-activated protein kinase (MAPK) pathway [20]. As shown in Fig. 5A, cells treated with a TGR-5 inhibitor showed a reduced degree of the bile acid-mediated HSC invasion. Moreover, while cells treated with DC increased Mcl-1 and COX-2 protein expression levels compared to those of the control group, a decreased expression of Mcl-1 and COX-2 proteins was observed in the group of LX-2 cells treated with a TGR-5 inhibitor in hypoxic conditions (Fig. 5B). These findings collectively indicate that Mcl-1 and COX-2 induction is due to transcriptional enhancement dependent on TGR-5 activation.

The selective inhibition of TGR-5 decreased the expressions of IL-8 under hypoxic conditions

To demonstrate the TGR-5 dependent induction of SASP (i.e. IL-8), LX-2 cells were treated with a TGR-5 inhibitor in the absence and in the presence of DC under hypoxic conditions. As shown in Fig. 6, while cells treated with a TGR-5 inhibitor in the absence of DC showed increased expression levels of IL-8, cells treated with a TGR-5 inhibitor in the presence of DC showed an attenuated degree of IL-8 expression levels. This result suggests that IL-8 induction is also due to the bile acid dependent TGR-5 activation.

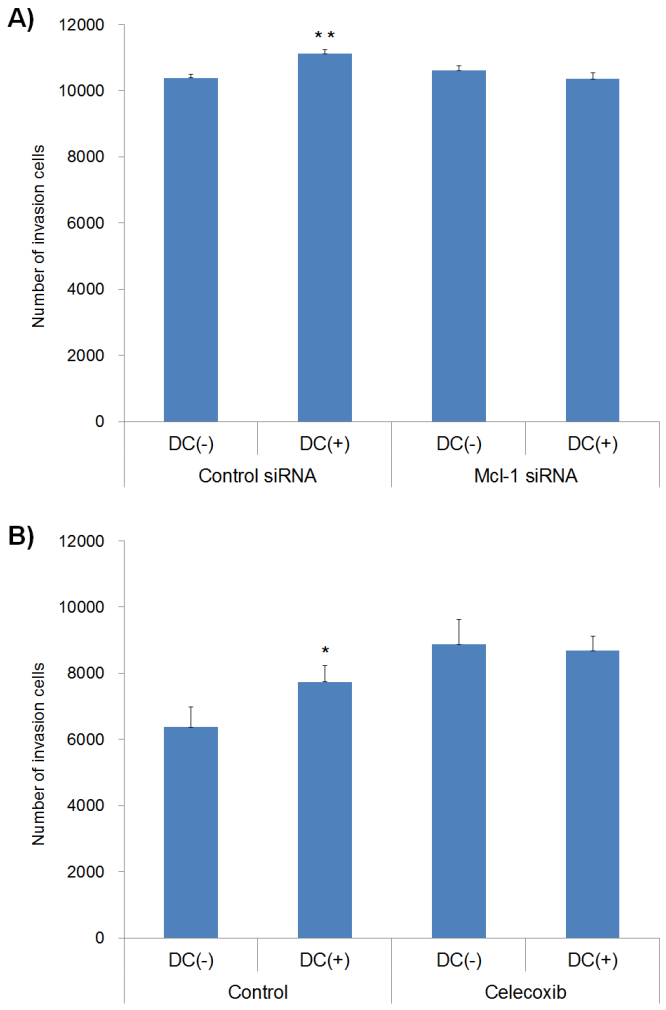

Figure 4. The selective inhibition of Mcl-1 and COX-2 and HSC invasion under hypoxic conditions. (A) LX-2 cells were transfected with Mcl-1 siRNA or not and treated with media (control) or DC (200 μM). (B) LX-2 cells were treated with/without the COX-2 inhibitor, celecoxib (20 μM), in the absence or presence of DC (200 μM). At the indicated time, invasion assays were performed, as instructed by the manufacturer. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

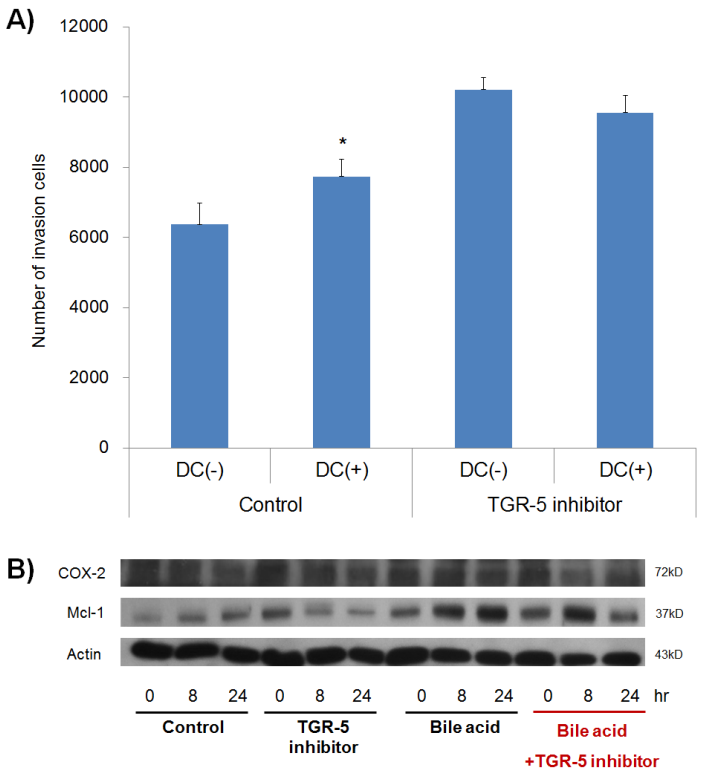

Figure 5. The selective inhibition of TGR-5 suppressed the bile acid-mediated HSC invasion and the protein expressions of Mcl-1 and COX-2 under hypoxic conditions. LX-2 cells were treated with/without the TGR-5 inhibitor, MDL-12,330A (10 μM), in the absence or presence of DC (200 μM). (A) Invasion ability was determined by invasion assay. (B) At each time point, cells were lysed and immunoblot analysis was performed for COX-2, Mcl-1, and actin. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

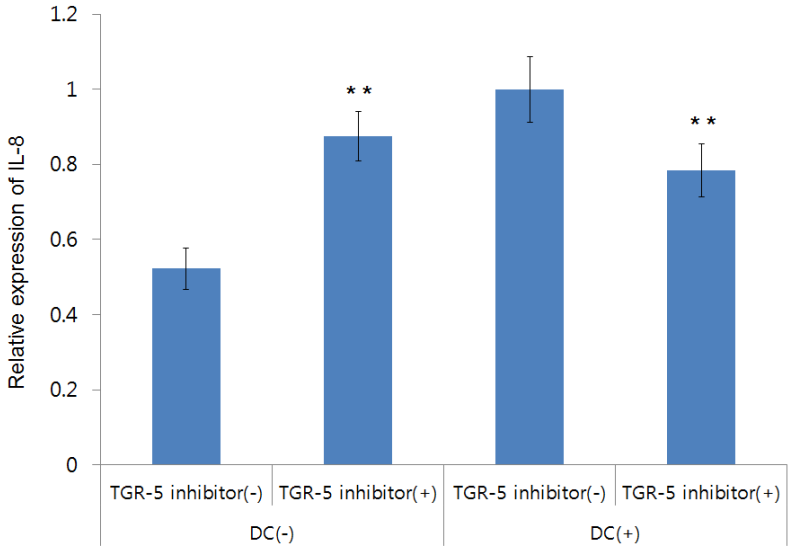

Figure 6. The selective inhibition of TGR-5 decreased the expressions of IL-8 under hypoxic conditions. LX-2 cells were treated with/without the TGR-5 inhibitor, MDL-12,330A (10 μM), in the absence or presence of DC (200 μM). IL-8 was quantitated using real-time PCR using SYBR green as fulorophore and a LightCycler.

*$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

Discussion

The remarkable and interesting findings of this study involve the TGR-5 dependent overexpression of Mcl-1 and COX-2 in bile acid-mediated HSC senescence under hypoxic conditions. As the rapidly growing literature indicates that activated HSCs and associated cellular senescence are associated with the progression of HCC [21-23], these results may provide new information for preventing the progression of HCC under hypoxic conditions by inhibiting bile acid-mediated HSC activation.

In the previous study, we demonstrated that bile acid increases COX-2 and Mcl-1 protein expression levels in HSCs, which function as anti-apoptotic signals in these cells against bile acid-induced apoptosis [5]. It was shown that bile acid was responsible for the induction of COX-2 expression in HSCs via multiple mechanisms including p42/44, p38 MAPK, and JNK signaling pathways. The inhibitions of p42/44, p38 MAPK or JNK resulted in the inhibition of COX-2 induction by bile acid exposure [5]. Activated p42/44 MAPK can directly phosphorylate and activate transcriptional factors, such as Elk-1 and Sap-1, and these factors conjoin to the serum-responsive element of the c-fos promotor, which then increases the expression of c-fos and c-jun and the binding activity of AP-1 (a transcriptional factor comprised of c-fos and c-jun protein complexes) [24,25]. The JNK signaling pathway is also responsible

for transcriptionally active AP-1 complexes [26]. The AP-1 complex transactivates the COX-2 promotor, which results in increasing the expressions of COX-2 mRNA and protein [27]; however, compared with the bile acid-mediated induction of COX-2 expression on the transcriptional level, our previous study showed that bile acid did not increase Mcl-1 mRNA levels, which suggests that increased Mcl-1 protein expression levels in HSCs by bile acid is due to a translational or post-translational process [5]. Raf-1, which is activated by bile acids, is assumed to be involved in the inhibition of Mcl-1 protein degradation as previously demonstrated in cholangiocytes [7]. Consistent with previous studies, our findings indicated that bile acids increase the invasion of HSCs as well as COX-2 and Mcl-1 protein expression levels in HSCs even under normoxic conditions; however, it remained unclear until now how hypoxia adjunctively affects the bile acid-mediated activation and invasion of HSCs.

It is well-known that hypoxia in local micro-environment plays an important role in the pathogenesis of different diseases, such as tumors. Hypoxia-inducible factor-1 (HIF-1) is the key transcriptional regulation factor, and recent data describe a possible role for HIF-1 in the modulation of apoptosis in addition to the role played by HIF-1 in the adaptation to hypoxia [28]. HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β. While HIF-1β is constitutively expressed, HIF-1α is

highly regulated by micro-environmental oxygenation. In normoxia, HIF-1α undergoes continuous degradation through oxygen-dependent uniquitination, maintaining a low concentration of HIF-1α; however, under hypoxia, HIF-1α is no longer modified and is thus stabilized, which then dimerizes with HIF-1β and enters the nucleus to bind with hypoxia-responsive elements (HRE) of target genes involved in angiogenesis, invasion and drug resistance [29]. Recently, it was reported that an Mcl-1 promotor contains an active HRE site and that HIF-1 specifically binds to the site to regulate the Mcl-1 expression under hypoxia, which protects cells against apoptosis [30]. In addition, it was reported that HIF-1 directly upregulates COX-2 expression and increases PGE2 production during hypoxia in colorectal tumor cells. This leads to the activation of the Ras-MAPK pathway, which may result in a positive feedback loop to retain an active pro-survival COX-2/PGE2 pathway during hostile micro-environmental conditions [31]. This hypoxia induced Ras-MAPK and COX-2/PGE2 pathway was also proven with HSCs in the previous study, which shows that MAPK phosphorylation stimulated by hypoxia is essential to HIF-1α activity. This includes the attenuation of HIF-1α uniquitination and the promotion of HIF-1α nuclear translocation, so that HIF-1α enters the nucleus to bind HRE and regulate cell survival in hypoxia [32]. Thus, regardless of the bile acid exposure, it is most likely that hypoxia itself

is also a strong inducer of Mcl-1 and COX-2 via HIF-1. However, it seems that the bile acid exposure had a greater effect on inductions of Mcl-1 and COX-2 than hypoxia since a decreased expression of Mcl-1 and COX-2 proteins was observed with a bile acid receptor blockage even in hypoxic conditions.

Cellular senescence is a process that occurs in normal cells in response to genotoxic stress, which acts through a checkpoint activation and stable cell-cycle arrest as a barrier to tumorigenesis [33]. Recent studies indicate that senescent cells are not silent cells, but develop a typical signature termed the SASP consisted mainly of inflammatory cytokines, such as IL-6 and IL-8, chemokines, and proteases, which actively influence surrounding cells by a paracrine manner [4]. Irreversible cell cycle arrest during cellular senescence is mediated by p21, a universal cell cycle inhibitor, which also contributes to the stability of cell cycle arrest long after the induction of senescence [34]. Thus, it plays a vital role in the induction and maintenance of cellular senescence and is also used as a cellular senescence marker. Recently, Yoshimoto et al. demonstrated that SASP of HSC induced by a bacterial product, DC, promotes obesity-associated HCC progression [35]. Although the mechanism of DC-induced HSC senescence has not been clarified, researchers have found that a number of p21-expressing senescent cells (HSCs rather than Kupffer cells) induced by DC in HCC

lesions produce proinflammatory cytokines, such as IL-6, GROα, and CXCL9, to promote obesity-associated HCC [35]. Moreover, in an esophageal stratified squamous epithelial model, the unconjugated bile acids (DC) significantly induced IL-8 production via two independent signaling pathways, p38 MAPK and protein kinase A pathway [36]. The other previous study revealed that IL-8 secreted by cervical carcinoma cells induced by hypoxia can stimulate the viability of cervical carcinoma cells in an autocrine dependent manner [37]. In the present study, a real-time PCR analysis showed that the levels of IL-8 were promptly increased when LX-2 cells were cultured under hypoxic conditions, especially with bile acid treatment. Moreover, the protein expression of p21 in HSCs was distinctly increased in the presence of bile acid under hypoxic conditions. In particular, TGR-5 inhibitor treatment reduced the bile acid-mediated HSC invasion as well as the Mcl-1, COX-2, and IL-8 expression levels in HSCs under hypoxic conditions. Therefore, our findings implicate that bile acid exposure under hypoxic conditions plays a pivotal role in inducing SASP in HSC and that bile acid receptors, such as TGR5, may contribute to bile acid-induced HSC senescence in the upstream pathway.

In conclusion, the present study demonstrates that the bile acid-mediated induction of HSC activation and the invasion by the SASP was due to TGR-5 dependent overexpression of Mcl-1 and COX-2,

which may lead to HCC progression under hypoxic conditions. Further studies are warranted to identify the exact mechanism of TGR-5 dependent HSC senescence and the detailed role of HSC senescence in hepatocarcinogenesis.

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국문초록

연구배경 및 목적: 활성화된 간성상세포는 간암의 성장과 이동을 조장하는 간 내 미세환경을 이루는 중요한 간질세포 중 하나이다. 또한, 이 과정에는 세포 노쇠 및 이와 관련된 다양한 염증성 분자그룹을 총칭하는 senescence-associated secretory phenotype (SASP)이 관여하는 것으로 알려져 있다. 따라서, 본 연구에서는 저산소조건에서 SASP와 관련하여 담즙산이 간성상세포의 활성화에 기여하는 영향을 알아보고자 하였다.

방법: Immortalized human stellate cell line인 LX-2 세포를 사용하여 정상산소조건 및 저산소조건에서 간성상세포의 활성화 및 침습 정도를 세포 침습 측정 및 웨스턴 블롯을 통해 평가하였다. 또한, SASP의 관여 여부를 확인하고자, IL-8 mRNA를 RT-PCR을 통해 정량화하였다.

결과: 담즙산은 정상산소조건에 비해 저산소조건에서 간성상세포의 침습성을 유의하게 증가시켰다. 또한, SASP의 중요한 인자 중 하나인 IL-8의 발현 증가도 확인하여 SASP가 간성상세포 침습성 증가에 영향을 미칠 것이라는 간접적인 결과를 확인하였다. 담즙산은 간성상세포 활성화의 지표인 α-SMA와 vimentin의 발현도 증가시켰다. 나아가 담즙산은 간성상세포

내부의 Mcl-1 및 COX-2의 발현을 증가시켰으며, Mcl-1 siRNA 핵내 주입을 통해 Mcl-1을 억제하거나 celecoxib를 통해 COX-2를 억제하였을 시에는 저산소조건에서 담즙산에 의한 간성상세포 침습성이 감소되는 결과를 확인하였다. 또한, 간성상세포 내 Mcl-1 및 COX-2의 발현 증가는 담즙산 수용체의 활성화에 의한 것으로 확인되었다.

결론: 저산소조건에서 담즙산에 의한 간성상세포의 침습성 증가는 SASP 및 담즙산 수용체 활성화에 의한 Mcl-1 및 COX-2 발현 증가에 의한 결과임을 확인할 수 있었다. 향후 간성상세포의 활성화 억제가 간암의 진행을 억제할 수 있는지를 탐구하기 위한 추가 연구가 필요할 것으로 사료된다.

주요어: hepatic stellate cell, bile acid, Cyclooxygenase-2, Mcl-1, hypoxia

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