Reversal of liver cancer-associated stellate cell-induced stem-like characteristics in SMMC-7721 cells by 8-bromo-7-methoxychrysin via inhibiting STAT3 activation

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Abstract. Hepatic stellate cells (HSCs) that are activated by human hepatocellular carcinoma (HCC) cells secrete a variety of cytokines, which are the main component of the HCC microenvironment. We aimed to determine whether 8-bromo-7-methoxychrysin (BrMC) could interfere in cross-talk of the human hepatic stellate cell line LX-2 and liver cancer stem-like cells (LCSLCs) to inhibit the characteristics of LCSLCs endowed with the capacity of sustaining human hepatocellular carcinoma (HCC) self-renewal and progression, and to identify its potential mechanism of action. We found that the levels of fibroblast activation protein (FAP) were augmented in LX-2 cells treated with the conditioned medium of LCSLCs (LCSLC-CM) compared to those cultured with routine medium, indicating that the LCSLC-CM can activate LX-2 cells to become liver cancer-associated stellate cells (LCAHSCs). Furthermore, sphere forming capability of SMMC-7721 cells was enhanced and stem cell-related protein expression was significantly increased following treatment with the conditioned medium of LCAHSCs (LCAHSC-CM). Moreover, the level of p-STAT3 was increased in LX-2 cells treated with LCSLC-CM and BrMC reduced expression of p-STAT3. Combination of BrMC and the selective inhibitor of STAT3 cucurbitacin I (JSI-124) synergistically suppressed the LCSLC characteristics in SMMC-7721 cells. Collectively, our data showed that BrMC inhibited the interaction between LX-2 cells and HCC-derived CSCs, and did so potentially through modulation of the STAT3 pathway. Future therapeutic strategies employing anti-CSC therapy should confirm the potential of cucurbitacin I (JSI-124) and BrMC as potent therapeutic agents.

Introduction

The fifth most common cancer globally is human hepatocellular carcinoma (HCC), and in the context of cancer-related mortality HCC ranks third as one of the leading causes (1,2). Moreover, traditional therapy remains disappointing. It is believed that HCC is sustained by liver cancer stem cells (LCSCs), which constitute a relatively small frequency of cells, and they do so through their ability to propagate highly heterogeneous progeny, multi-drug resistance to chemotherapeutic agents and a high capacity for cellular proliferation (3). A number of studies have suggested that LCSCs can be identified by several cell surface antigens including CD133, CD44, and ALDH1 (4-6). Furthermore, CD133+ cells that are isolated from HCC cells display an enhanced capacity for malignant transformation in vivo. These studies indicate LCSCs as the basis of HCC. However, emerging evidence shows that cancer onset and progression was not only determined by tumor cells but was also affected by the local microenvironment (7,8).

It has been reported that almost 80% of hepatocellular carcinoma (HCC) resulted from chronic hepatitis and cirrhosis caused by inflammation and fibrosis (9). Hepatic stellate cells (HSCs), which are the main liver stromal cells, can transform into a myofibroblast-like phenotype from a quiescent state during chronic liver injury (10,11). An important cellular source of hepatic-derived cytokines (e.g., TGF-β, PDGF, HGF, PFG, and VEGF) is secreted by HSCs that constitute the main component of the local liver cancer microenvironment. Additionally, HSC activation might play an important role in inflammation and fibrosis, even during tumor metastasis (9). It has been shown recently that malignant transformation in HSC is critical in reprogramming cells that transform the physiologically normal vitamin-A storage capacity of HSC to a remodeled extracellular matrix phenotype (11), which then provides a tumorigenic environment that is compatible for HCC. Additionally, studies have shown that cross-talk of hepatocytes and HSC can generate a permissive inflammatory...
microenvironment to promote HCC development (12), and the interaction of hepatocytes and HSCs leads to tumor metastasis, proliferation and chemotherpay resistance (13). However, whether LCSCs activate HSC in a way that promotes HCC progression remains to be fully explored.

Signal transducer and activator of transcription-3 (STAT3), a transcription factor for cytokine signaling, is constitutively activated in numerous cancer types, including prostate cancer, breast cancer, leukemia, brain tumors, and lung cancer (14-18). Niu et al reported that STAT3 mutations induced cellular transformation and tumor formation in vivo and that activation of STAT3 signaling further inhibited p53 transcriptional activity, fulfilling the definition of an oncogene (19). In addition, it is reported that activated STAT3 could promote LCSCs (20). Moreover, activation of STAT3 in hepatic stellate cells promoted their survival and proliferation, thereby contributing to liver fibrogenesis (21,22). However, the mechanism of the STAT3 signaling pathways to interrupt cross-talk of LCSCs and HSC and the possible therapeutic targets involved in HCC need further investigation.

Chucubnicin I (JSI-124) is a cell permeable, triterpenoid compound that was shown to specifically suppress tyrosine phosphorylation of STAT3 (23). In the current study, we hypothesized that JSI-124 would be useful to block cross-talk of LCSCs and HSC by inhibiting activation of STAT3.

Chrysin (5,7-dihydroxyflavone, ChR), a natural widely distributed flavonoid, has been shown to possess promising effects on the inhibition of proliferation and induction of apoptosis in a variety of cancer cells (24,25). By using a STAT3-specific inhibitor, JSI-124, Lirdprapamongkol et al showed that ChR overcame TRAIL resistance of cancer cells through inhibiting STAT3 phosphorylation (26). Furthermore, Lin et al reported that ChR suppresses IL-6-induced angiogenesis through modulation of the STAT3 signaling pathway (27). In a previous study, we succeeded in synthesizing 8-bromo-7-methoxychrysin (BrMC) based of the lead compound ChR (28). Compared to ChR, BrMC has stronger effects of inhibition of proliferation and induction of apoptosis on colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 (28,29). As previously described, BrMC can inhibit the proliferative activity of glioma stem-like cells, and target to inhibit the characteristic of LCSCs (30,31). Thus, our aim was to analyze if BrMC can affect cross-talk of liver cancer stem cells and HSC to reduce the activation of HSC and inhibit the properties of LCSCs by suppressing STAT3.

Materials and methods

Reagents. Dulbecco’s modified Eagle’s medium (DMEM) and DMEM/F12 medium, Trypsin-EDTA, FBS and Penicillin-streptomycin were from Gibco (Grand Island, NY, USA). All cell culture ware was from Corning Life Sciences (New York, NY, USA). Monoclonal anti-α-SMA-Cy3 antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal anti-FAP-α, polyclonal anti-E-cadherin, polyclonal anti-N-cadherin, monoclonal anti-CD33, monoclonal anti-CD44, monoclonal anti-stat3, and polyclonal anti-ALDH1 antibody were obtained from Abcam (Hong Kong, China). The STAT3 inhibitor, JSI-124, was obtained from Sigma-Aldrich, and was prepared by dissolving in DMSO to a stock concentration of 100 μmol/l. JSI-124 was additionally diluted in culture medium to required final concentrations immediately prior to use. BrMC was synthesized as previously described (28). All other experimental reagents used in this study were obtained from Sigma-Aldrich, unless indicated otherwise in the text.

Cell culture and sphere formation assay. The Chinese Academy of Sciences (Shanghai, China) provided the human liver cancer SMMC-7721 cell line. The human immortalized HSCs (LX-2) was obtained from Bogu Biotechnology Co., Ltd. (Shanghai, China). Cells were routinely passaged in complete DMEM that was supplemented with 10% fetal bovine serum (FBS), and antibiotics. Cell cultures were incubated at 37°C in an atmosphere of 5% CO2 in air.

Suspensions of single-cells were seeded into ultra low attachment 6-well plates (Corning Life Sciences) at a density of 3,000 cells/ml in stem cell-conditioned medium. Culture suspensions were passaged every five days when spheroid diameters were at least 50 μm. Colonies were then scored under ten independent fields of view by light microscopy (Olympus, Tokyo, Japan). The efficiency of sphere formation was calculated by dividing the total sphere number that formed by the number of the total viable cells that were seeded multiplied by one hundred.

Preparation of conditioned medium from LCSCs and LCAHSCs. To prepare LCSCL-CM, suspension culture and stem cell-conditioned medium amplification of LCSCs was performed using ultra low-adhesion 6-well plates. Spent culture media was removed 24 h later, and this was filtered (0.22 μm) and stored at -80°C until further use.

Immunoﬂuorescence. LX-2 cells were cultured on coverslips, washed with PBS, and fixed in 4% paraformaldehyde, following which, 0.1% triton X-100 for 4 min was used to permeabilize the cells. Next, monoclonal anti-α-SMA-Cy3 or polyclonal anti-FAP-α antibody was added for 45 min or 1 h at 37°C in the dark. Later, Alexa Fluor 488 anti-rabbit (Molecular Probes, 1:500, 1 h, RT) and DAPI [1:100, 10 min, room temperature (RT)] were incubated with the coverslips, which were then mounted for visualization and quantification. Images were collected by fluorescence microscopy (Olympus).

Western immunoblot analysis. Previously described procedures were used for preparing whole cell lysates and the western immunoblot procedure (32). The primary antibodies used in this procedure were monoclonal anti-α-SMA antibody, polyclonal anti-FAP-α, polyclonal anti-E-cadherin, polyclonal anti-N-cadherin, monoclonal anti-CD33, monoclonal anti-CD44, monoclonal anti-stat3, and polyclonal anti-ALDH1 antibody were incubated with the coverslips, which were then mounted for visualization and quantification. Images were collected by fluorescence microscopy (Olympus).
Heights, IL, USA). The autoradiographed images were scanned to permit semi-quantitative densitometric analysis using UN-SCAN-IT software program (Silk Scientific).

**Statistical analyses.** Representative data described in this report are the product of at least three independent observations, unless otherwise indicated. The data were analyzed using analysis of variance followed by Dunnett's test for pairwise comparison. An asterisk indicates that the experimental values were significantly different from values at an α value of P<0.05.

**Results**

**Characteristics of liver cancer stem-like cells derived from SMMC-7721 cells.** To investigate whether the third generation sphere forming cells (SFCs) of SMMC-7721 cell line possess properties of liver cancer stem-like cells (LCSLCs), sphere formation assay was performed. Furthermore, the level of CD133, CD44, ALDH1 expression, which are known as markers of stem cells, were determined. Consistent with a previous study (30,31), the third generation of SMMC-7721 SFCs have stronger capability of proliferation and its sphere forming rate increases compared to parental cells (Fig. 1A and B). In addition, the expression of CD133, CD44, and ALDH1 is elevated in the third generation of SFCs compared to parental cells (Fig. 1C). These results indicated that the third generation SMMC-7721 SFCs have properties of LCSCs.

**Generation of liver cancer associated-stellate cells derived from human hepatic stellate cell line LX-2.** Human hepatic stellate cell line LX-2 was exposed to conditioned medium from liver cancer stem-like cells (LCSLC-CM) for 24 h to generate liver cancer associated-hepatic stellate cells.
Figure 2. LX-2 cells are activated by LCSLC-CM. (A) Representative immunofluorescence images showing α-SMA and FAP expression, previously exposed to LCSLC-CM for 24 h, using serum-free medium as a control. Western blot expression of α-SMA (B) and FAP protein (D). To control for equal loading of protein samples, we used β-actin expression. (C and E) Densitometry analysis to quantify the expression of α-SMA and FAP protein (*P<0.05).

Figure 3. LCSLC-CM treated with BrMC reduces pathologically activated LX-2 cells. (A) Treated with LCSLC-CM containing different concentrations of BrMC (0.0, 5.0, 10.0, 20.0 µmol/l), representative images of the expression levels of both α-SMA and FAP in LX-2 cells are shown. Western blot of the expression of α-SMA (B) and FAP (D) in LX-2 cells, and β-actin used as loading control. Densitometry analysis to quantify the expression of α-SMA (C) and FAP (E) protein, respectively (*P<0.05).
(LCAHSCs), and verify the capacity of LCSLC-CM induce LX-2 cell pathologic activation. As shown in Fig. 2A, LX-2 cells treated with LCSLC-CM induced significant changes in fibroblast activation protein (FAP) expression, but no visible changes in α-smooth muscle actin (α-SMA) expression. This result is consistent with western blot analysis.

**Effects of 8-bromo-7-methoxychrysin on activation of LX-2 cells by LCSLC-CM.** We previously described that BrMC can inhibit several forms of cancer (31,33). In the current study, to analyze if BrMC can affect cross-talk of LCSLCs and HSCs to block the activation of HSC, we measured the expression of α-SMA and FAP by immunofluorescence and western blotting in LX-2 cells after being cultured with LCSLC-CM containing various concentrations of BrMC (0.0, 5.0, 10.0, 20.0 µmol/l). As shown in Fig. 3A, immunofluorescence showed that BrMC had no effect on the expression of α-SMA, but it reduced the level of FAP expression in a dose-dependent manner. Consistent with the results of immunofluorescence, western blot analysis indicated downregulation of FAP (Fig. 3D and E), but not α-SMA (Fig. 3B and C).

**Conditioned medium from liver cancer associated-stellate cells contributes to characteristics of LCSLCs derived from SMMC-7721 cells.** To evaluate the effects of LCAHSCs on self-renewal capability and cancer stem cell marker expression of SMMC-7721 cells and LCSLCs derived from SMMC-7721 cells, respectively; we collected conditioned medium from LCAHSCs (LCAHSC-CM), and the cells were exposed to LCAHSC-CM for 24 h. Then sphere formation assay and western blotting were performed. As expected, treatment with LCASC-CM enhanced the sphere forming ability of SMMC-7721 cells (Fig. 4A and B). The level of CD133 (Fig. 4C and F), CD44 (Fig. 4D and G) and ALDH1 (Fig. 4E and H) in the cells treated with LCAHSC-CM were significantly increased compared to untreated cells.

**BrMC reverses the characteristics of LCSLCs induced by LCAHSC-CM.** Our findings showed that LCAHSC-CM can promote the characteristics of LCSLCs and BrMC can inhibit activation of LX-2 cells. Thus, we investigated whether BrMC inhibits the characteristics of LCSLCs induced by LCAHSC-CM. Sphere forming assay indicated that BrMC inhibits self-renewal capability of SMMC-7721 cells in a dose-dependent manner, and the sphere forming rate was significant reduced when treatment with 20 µmol/l BrMC (Fig. 5A and B). The markers of cancer stem cells (including CD133, CD44 and ALDH1) were also reduced by BrMC in a dose-dependent manner (Fig. 5C).

**BrMC inhibits the phosphorylation of STAT3 in LX-2 cells induced by LCSLC-CM.** It was reported that IL6/STAT3 axis
activated LX-2 cells (34). Thus, we studied whether STAT3 was responsible for LX-2 cells activation induced by LCSLC-CM, and if BrMC can decrease the phosphorylation of STAT3 to inhibit LX-2 cells activation. The results in Fig. 6A show that compared to serum-free DMEM/F12 medium, LCSLC-CM induced phosphorylation of STAT3 in LX-2 cells, but not total STAT3 protein expression. LCSLC-CM from LCSLCs treated with different concentrations of BrMC (0.0, 5.0, 10.0, 20.0 µmol/l), showed phosphorylation of STAT3 decreased in a dose-dependent manner, and no effect on the level of STAT3 expression (Fig. 6B).

JSI-124 treatment reverses the characteristics of LCSLCs induced by LCAHSC-CM. The above studies showed that LCSLC-CM induced the phosphorylation of STAT3 in LX-2 cells and LCAHSC-CM contributed to the characteristics of LCSLCs. To explain the mechanism that activated the LX-2 cell promoted features of LCSLCs derived from SMMC-7721
cells, STAT3 inhibitor JSI-124 was used. Our findings showed that when added to LCSLC-CM treated with JSI-124, the phosphorylation of STAT3 in LX-2 cells was significant reduced, but it had no effect on the expression of STAT3 compared with LCSLC-CM-treated LX-2 cells (Fig. 7A and B). Then we treated LCSLCs and SMMC-7721 cells with LCAHSC-CM containing different concentration of JSI-124 (0.0, 10.0, 30.0, 100.0 nmol/l) to determine their stem cell marker expression and sphere formation, respectively. Sphere formation induced by LCAHSC-CM was inhibited by JSI-124 in a dose-dependent manner (Fig. 7C). Western blotting showed that the level of cancer stem cell markers (CD133 (Fig. 7E), CD44 (Fig. 7G) and ALDH1 (Fig. 7I) were decreased after treated with JSI-124. In conclusion, JSI-124 inhibited the characteristics of LCSLCs induced by LCAHSC-CM.

BrMC and JSI-124 synergistically inhibit properties of LCSLCs induced by LCAHSC-CM. The above studies showed that BrMC reversed the characteristic of LCSLCs through inhibiting STAT3. Thus, we used STAT3 inhibitor JSI-124 (10 nmol/l) and BrMC (5.0 µmol/l) alone or combined to administer LCSLCs and to obtain LCSLC-CM containing BrMC or JSI-124 or both, which was used to culture LX-2 cells for 24 h and collect LCAHSC-CM. Next, the STAT3 in LX-2 was determined by western blotting. We validated the role of BrMC and JSI-124 in the inhibition of the characteristics of LCSLCs. Our data showed that the presence of BrMC and JSI-124 was sufficient to reduce phosphorylation of STAT3, which indicated that BrMC and JSI-124 synergistically inhibited LX-2, which permitted them to be activated by LCSLC-CM (Fig. 8A). Then we collected LCAHSC-CM that contained BrMC or JSI-124 alone or combined to measure properties of LCSLCs. The sphere forming assay showed that combined BrMC (5.0 µmol/l) and JSI-124 (10 nmol/l) significantly inhibited the ability of sphere forming compared to treating with BrMC and JSI-124 alone (Fig. 8C). Then, western blotting was carried out to detect the expression of stem-cell markers (CD133, CD44 and ALDH1). The results indicated that combination of BrMC (5.0 µmol/l) and JSI-124 (10 nmol/l) had a significant impact on the levels of CD133, CD44 and ALDH1 expression compared to BrMC or JSI-124 alone (Fig. 8E).

Discussion

LSCSs are considered the key factors of HCC progress. In addition, the tumor microenvironment plays an important role during carcinogenesis. Carcinoma-associated fibroblasts (CAFs) are one of the most crucial components of the HCC microenvironment. In this study, we put forward that human hepatic stellate cell line LX-2 can be activated to a myofibroblast-like phenotype through STAT3 pathway, and activated LX-2 cells in turn promote the characteristics of LCSLCs. Moreover, BrMC affected cross-talk of LCSLCs and LX-2 cells to reduce the activation of HSC and then reversed the characteristics of LCSLCs. CAFs are thought to be activated, which is characterized by the expression of α-SMA and FAP (35-37). Moreover, activated fibroblasts in tumor tissues are considered as CAFs. In our present study, we proved that LCSLCs derived from SMMC-7721 cells had interaction with LX-2 cells, and made LX-2 cells pathologically activated with a myofibroblast-like phenotype, named LCAHSC. Our results are consistent with the identification of activated CAFs as these cells expressed α-SMA and FAP, whereas cells treated without LCSLC-CM
did not express FAP (Fig. 2). Noteworthy, the LX-2 cells treated without LCSLC-CM also express α-SMA, which suggests that these cells exist in an activated state under cell culture conditions. We found that the conditioned medium from pathologically activated LX-2 cells significantly promoted the characteristics of LCSLCs of the SMMC-7721 cell line (Fig. 3).

STAT3, a transcription factor mediating various cellular processes and participating in cellular transformation, is aberrantly activated in numerous cancer types, including...
HCC. Since the persistent activation of STAT3 promotes tumor cell proliferation and survival, contributing to tumor progression, abrogation of STAT3 signaling is emerging as a potential cancer therapy strategy (38). We found that the level...
of p-STAT3 was greater in LX-2 cell treated with LCSLC-CM than control (Fig. 6A). JSI-124, an inhibitor of the JAK/STAT pathway, could effectively block STAT3 signaling in a dose-dependent manner, and further inhibited the characteristics of liver cancer stem-like cells induced by LCAHSC-CM (Fig. 7). To our knowledge, this is the first study to demonstrate that the STAT3 plays an important role in the interaction of LCSLCs and LX-2 cells.

8-bromo-7-methoxychrysin (BrMC) was synthesized previously based of the lead compound ChR (28). In addition, BrMC has strong effects of inhibition of proliferation and induction of apoptosis on various types of cancer (39,40). In the current study, we demonstrated that BrMC significantly reduced the activation of LX-2 induced by LCSLC-CM, and inhibited the properties of LCSLCs induced by LCAHSC-CM (Fig. 4). Of note, the level of p-STAT3 in LCAHSC was greatly decreased after treated with BrMC (Fig. 6C), suggesting BrMC could inhibit the activation of LX-2 induced by LCSLC-CM through attenuating STAT3 activation, and then reversed the characteristics of LCSLCs induced by LCAHSC-CM.

Our data also showed that BrMC (5.0 µmol/l) and JSI-124 (10 nmol/l) synergistically inhibited the expression of p-STAT3 in LX-2 cells, and the expression levels of α-SMA and FAP decreased in response to BrMC (5.0 µmol/l) and JSI-124 (10 nmol/l), which support the possibility that inhibition of the STAT3 pathway may significantly block transformation of LX-2 cells from a quiescent state into a myofibroblast-like phenotype. Then, combination of BrMC (5.0 µmol/l) and JSI-124 (10 nmol/l) significantly decreased the sphere formation and the expression levels of stem cell markers (CD133, CD44 and ALDH1) (Fig. 8). Taken together, our findings indicated that combined BrMC and JSI-124 significantly inhibited cross-talk of LX-2 cells and LCSLCs probably through suppressing the activation of STAT3.

Compared with LCSCs, the relevance between LCSCs, hepatic stellate cells, and STAT3 has been less clearly identified. A recent report demonstrated that IL6/STAT3 axis was sufficient for transdifferentiation of quiescent fibroblasts to CAFs (34). It was reported that cross-talk between hepatoma cells and activated HSCs engendered a permissive inflammatory microenvironment that drives HCC progression (12). In this study, we discovered that human hepatic stellate cell line LX-2 could be activated by LCSLC-CM through STAT3 signaling pathway, which was inhibited by BrMC. However, the establishment of co-culture model may be required to further evaluate the therapeutic possibilities of combination with JSI-124 and BrMC.

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