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CTGF secreted by mesenchymal-like hepatocellular carcinoma cells plays a role in the polarization of macrophages in hepatocellular carcinoma progression

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ABSTRACT

M2 macrophages play critical roles in the progression of hepatocellular carcinoma (HCC), and they are associated with poor outcomes. TGF-β-induced epithelial-mesenchymal transition (EMT) has been shown to be critically important to cancer cell dissemination in HCC. However, the relationship between stromal-like HCC cells and M2 macrophages formation is not clear. Here, we interrogated the molecular link between mesenchymal-like HCC cells and the formation of M2 macrophages. We demonstrated that mesenchymal-like HCC cells secrete connective tissue growth factor (CTGF) to polarized macrophages. Reciprocally, Chemokine ligand 18 (CCL18) from M2 macrophages promotes HCC progression.E Furthermore, CTGF and CCL18 were increased significantly in HCC compared to adjacent normal liver tissues. In summary, our study discovered a positive feedback loop between CTGF and CCL18 in HCC metastasis. Targeting CTGF or CCL18 might provide beneficial effects for the clinical treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed malignancies, especially in Asia [1]. HCC is difficult to treat, and it can be lethal if not diagnosed early. Because of the high rate of intra- or extra-hepatic metastasis, HCC patients have an unfavourable prognosis and high recurrence [2]. It is well established that the tumour microenvironment plays an important role in tumour development and metastasis [3]. M2 macrophages, the most abundant immune-related stromal cells in the tumour microenvironment, directly affect tumour cell growth, extracellular remodelling and neoangiogenesis [4]. M2 macrophages are abundant in solid tumours, but they play different roles in different cancers [5]. Tumour progression is negatively associated with the density of macrophages in brain, liver, breast, kidney and bladder cancer. Oppositely, tumour progression is positively associated with the density of macrophages in colorectal cancer. However, it is not yet known how M2 macrophages are formed in HCC.

Epithelial-mesenchymal transition (EMT) is a process that is closely associated with tumour metastasis in HCC and other cancers, as well as being involved in immunosuppression and drug resistance [2,6,7]. Recent studies have shown that mesenchymal-like breast cancer cells secrete granulocyte macrophage colony-stimulating factor (GM-CSF) to activate macrophages to a TAM-like phenotype [8], and CCL18 from M2 macrophages induces cancer cell EMT [8–11]. Moreover, in glioblastoma multiformes (GBMs), glioma stem cells (GSCs) secrete POSTN to recruited TAMs to promote the progression of GBMs [12]. Tumour-associated macrophages produce interleukin 6 and signal via STAT3 to promote the expansion of HCC stem cells [13]. Blockading IL6 signalling with tocilizumab inhibits the M2 macrophage-stimulated activity of HCC stem cells.

CTGF, also known as CCN2 and NOV2, is a member of the CCN cysteine-rich family of proteins. It is also a potent regulator of endothelial cell adhesion, proliferation, migration, angiogenesis and the modulation of extracellular matrix deposition, and it possesses a variety of functions both in vivo and in vitro [14]. CTGF is overexpressed in mesenchymal-like SMMC-7721 hepatocellular carcinoma cells and fibrotic human liver [7,15], and it enhances Wnt signalling in hepatocytes to contribute to liver fibrosis [16]. The expression of CTGF in HCC tumour cells correlates with poor clinical prognosis [17]. Moreover, CTGF was detected in blood samples from HCC patients, and higher serum CTGF levels led to reduced survival [18]. CTGF was associated with alternatively

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activated M2 type macrophages in chronic kidney allograft injury (CAI), which promotes the development of CAI [19]. Moreover, CTGF mediates the regulation of pancreatic inflammation. A selective inhibitor of TGF-β receptor type I-LY2109761 strongly reduced tumour progression by inhibiting the production of CTGF in HCC tumours [20]. A human monoclonal antibody against CTGF, which has been used in preclinical studies of pancreatic cancer, could inhibit tumour growth and metastasis [14,21] and anti-CTGF therapy can reduce migration and peritoneal adhesion of HGSOC cell lines with high CTGF expression [22].

In this study, we observed that mesenchymal-like HCC cells could secrete connective tissue growth factor (CTGF) to polarized macrophages. The relationship between mesenchymal-like HCC cells and M2 macrophages in HCC progression was observed. The key regulators of CTGF and CCL18 were further validated by qRT-PCR in HCC tissues and adjacent normal liver tissues, revealing that they were upregulated in tumour cells. These results demonstrated that CTGF and CCL18 could be potential therapeutic targets for the treatment of HCC.

2. Materials and methods

2.1. Isolation of monocytes

Peripheral blood monocytes (PBMCs) were isolated by Ficoll-Paque PREMIUM (GE Healthcare) from hepatocellular carcinoma cancer patients. First, 2 ml of defibrinated or anticoagulant-treated blood was mixed with an equal volume of 1640 medium (GIBCO) at 18 °C to 20 °C to a final volume of 4 ml, then the tube was inverted several times. Second, Ficoll-Paque PREMIUM (3 ml) was added to a centrifuge tube and the diluted blood samples (4 ml) were carefully layered onto Ficoll-Paque PREMIUM. The mixture was centrifuged at 4000*g* for 30 to 40 min before drawing off the upper layer containing plasma and platelets with a sterile pipette and leaving the layer of mononuclear cells undisturbed at the interface. Cells were washed three times with Hanks solution and resuspended in Dulbecco's modified Eagle's medium (HyClone) containing 10% foetal bovine serum (Biowest). On the following day, the media was replaced and the peripheral blood mononuclear cells (PBMCs) were collected.

2.2. Collection of culture supernatant

SMMC-7721 hepatocellular carcinoma cells were treated with 10 ng/ml of recombinant TGF-β (R&D Systems, Minneapolis, MN) for 7 days with TGF-β replenishment every 2 days. When those cells those had grown to 80% of confluence, they were washed with PBS and the medium was replaced. Three days later, the conditioned medium (CM) was collected by low-speed centrifugation to remove dead cells, then filtered through a 0.45 μm filter and aliquoted into small EP tubes before being frozen at −20 °C. Thirty percent CM was used to stimulate PBMCs for 7 days to obtain macrophages.

2.3. Cell cultures and treatment

SMMC-7721 hepatocellular carcinoma cells were obtained from the Chinese Academy of Sciences Cell Bank and grown in Dulbecco's modified Eagle's medium (HyClone) with 10% foetal bovine serum (Biowest) at 37 °C in an atmosphere containing 5% CO2. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood and cultured in the same conditions. For in vitro activation, peripheral blood mononuclear cells (PBMCs) were treated with 50 ng/ml CTGF for 7 days.

2.4. RNA extraction and quantitative PCR with reverse transcription (RT-qPCR)

Extraction of total RNA was done using TRIZOL (Invitrogen), and cDNA was generated using M-MLV reverse transcriptase (Invitrogen) following the manufacturer's instructions with random primers. RT-qPCR was performed using SYBR® Green (Takara, Dalian, China) in the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA). The gene-specific primers are shown in Tables 1 and 2. The real-time PCR reactions were performed in triplicate. β-actin was employed as an endogenous control. The relative expression was calculated using the comparative Ct method.

2.5. Western blot analysis

SMMC-7721 hepatocellular carcinoma cells that had been treated or not treated with TGF-β were harvested in RIPA buffer (Beyotime, Jiangsu, China) containing a protease inhibitor cocktail (Calbiochm, Billerica, MA, USA) and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was used as the total cell lysate. Lysates were added to 5× SDS-PAGE (Beyotime, Jiangsu, China) and boiled at 100 °C for 10 min. Identical quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membranes. The membranes were blocked for 1 h at room temperature in PBS containing 5% non-fat dry milk before being incubated at 4 °C in PBS-T containing the primary antibody overnight. After an incubation with antibodies specific for SLUG (Abcam), vimentin (Cell Signaling Technology, Boston, USA), ZO-1 (Abcam) and β-actin (Sigma-Aldrich), the blots were incubated with IRdye 700-conjugated goat anti-mouse IgG (Li-Cor Biosciences Inc., Lincoln, NE) and IRdye 800-conjugated goat anti-rabbit IgG (Li-Cor Biosciences Inc.). The signal was then detected using an Odyssey infrared scanner (Li-Cor Biosciences Inc.). β-actin was used as a loading control for western blots.

2.6. GEO dataset analysis

To explore whether secretion factors of mesenchymal-like HCC cells have the ability to activate macrophages, we used our previous microarray results (GSE54797) comparing TGF-β treated and untreated cells to find the regulatory factors that could be influencing macrophages. The primers and the expression of secreted factors between TGF-β treated and untreated cells are presented in Table 1. To determine the expression of CTGF and CCL18 in hepatocellular carcinoma, the clinical datasets in the Open-Access NCBI-GEO (https://www.ncbi.nlm.nih.gov/) were utilized. We used GSE39791 and GSE54236 to analyse gene expression. The expression data for all genes were obtained from single colour hybridization of human RNAs. For the whole genome microarray data sets, the A_23_P19663 (CTGF) and A_23_P55270 (CCL18) probes were analysed.

2.7. Wound healing migration assay

The wound healing migration assays were performed as previously described [6]. To investigate the effect of CCL18 on SMMC-7721 hepatocellular carcinoma cells migratory speed, SMMC-7721 hepatocellular carcinoma cells were grown in DMEM containing 10% foetal bovine serum to 90% confluency. The medium was then removed and a scratch was made through the layer of cells. Cells were washed with PBS twice and the medium was replaced with fresh medium containing 20 ng/ml CCL18. Cells were photographed at 0 h, 12 h and 24 h under phase-contrast microscopy to estimate the closure of the scratch.