Cancer Letters 404 (2017) 19–28

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Peri-tumor associated fibroblasts promote intrahepatic metastasis of hepatocellular carcinoma by recruiting cancer stem cells

Jinghua Jiang [1], Fei Ye [1], Xue Yang [1], Chen Zong, Lu Gao, Yang Yang, Qiudong Zhao, Zhipeng Han*, Lixin Wei

Department of Tumor Immunology and Gene Therapy Center, Shanghai Eastern Hepatobiliary Surgery Hospital, Shanghai, 200438, China

ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form
28 June 2017
Accepted 5 July 2017

Keywords:
Fibroblasts
Intrahepatic metastasis
Cancer stem cells
IL-6
CCL2
CXCL8

ABSTRACT

Fibroblasts have been reported to play an important role in hepatocellular carcinoma (HCC). However, the role of fibroblasts have not been fully understood. Conditioned medium collected from human peri-tumor tissue-derived fibroblasts (CM-pTAFs) showed high metastasis ability than human HCC tissues-derived fibroblasts (CM-TAFs). To determine what component was secreted from fibroblasts, we used Bio-Plex analysis system and compared the factors secreted from CM-pTAFs and CM-TAFs, found a series of up-regulated cytokines in the CM-pTAFs, including IL-6, CCL2, CXCL1, CXCL8, SCGF-β, HGF and VEGF. Pretreatment of IL-6 inhibitor Tocilizumab could inhibit metastasis the HCC cell treated with CM-pTAFs *in vitro* and *in vivo*. The expression of CCR2 and CXCR1 were up-regulated after CM-pTAFs treatment in HCC cell line SMMC-7721. Flow cytometric analysis experiment showed that most CCR2 or CXCR1 positive cells were also EpCAM positive. *In vitro* studies also showed that CM-pTAFs could increase stemness of SMMC-7721. In addition, neutralization of SCGF-β and HGF could significantly reduce metastasis and viability of cancer stem cells treated with CM-pTAFs. Taken together, these results indicated that the peri-tumor tissues derived fibroblasts may promote development of HCC by recruiting cancer stem cells and maintaining their stemness characteristic.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and also is a primary malignancy of the liver cancer [1–4]. Despite numerous efforts, hepatic resection represents an effective therapeutic strategy that largely improves survival. However, most patients showed recurrence within 5 years, and their prognosis is poor [1,5–7]. Therefore the well-understanding of the mechanism for progress of HCC is indispensable.

Most malignancies are composed of a subset of populations called cancer stem cells (CSCs). CSCs maintain self-renewal and multi-lineage differentiation property and are found at the central echelon of cellular hierarchy present within tumors. These cells maintaining their malignant potential, alteration of genomic integrity, epigenetic identity and the expression of specific surface protein profiles. As CSCs are highly resistant to chemotherapeutics, they are thought to be a crucial factor involved in tumor relapse and superficially appear as the ultimate therapeutic target [8].

CSCs found in the tumor microenvironment (TME), play a critical role in cell invasion and metastasis, progress of HCC is multiple steps and complicate phenomenon [9]. In this environment, fibroblasts play important role during cancer progress as one of the main member of stoma cells [10–12], but the critical role of fibroblast is still controversial. In most studies of cancer, tumor associated fibroblasts (TAFs) has been emphasized with attention being given to a significant modifier of cancer evolution [13,14], and they promote the tumorigenesis [15,16], progression [17], invasion [15,17,18], and chemoresistance [19,20] of cancer cells by various mechanisms. But there are no any evidence indicate that how peri-tumor tissues derived fibroblasts (pTAFs) affect to HCC present, growth, recurrence.

Here, we demonstrated that the pTAFs have more potential to accelerate the metastasis of HCC cells compared to TAFs. To verity the cytokines in the conditioned medium (CM) which collected from two kind of fibroblasts, we used Bio-Plex analysis system to compare pTAFs and TAFs. And found that the IL-6, CCL2, CXCL1, CXCL8, SCGF-β, HGF and VEGF was high expressed in CM-pTAFs

* Corresponding author.
E-mail address: hanzhipeng0311@126.com (Z. Han).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2017.07.006
0304-3835/

than CM-TAFs. Next, inhibition of IL-6 receptor before the CM-pTAFs treatment could decreased the metastasis of HCC cells *in vitro* and *in vivo*. Because the IL-6 is the one of the major inflammatory factor, this result demonstrated again how important is the inflammation during tumorigenesis and cancer metastasis. Additionally, we also found that the stem cell marker-EpCAM positive cells were also CCR2 and CXCR1 (the receptors of CCL2 and CXCL8 respectively) positive. Since the SCGF-β and HGF also help to maintain the stemness and viability of CSCs, these results indicated that the pTAFs accelerated metastasis of HCC, by recruiting CSCs from tumor to peri-tumor tissues.

Materials and methods

Liver tissues of HCC patients

A total of 6 patients with HCC who accepted clinical surgery therapy in the Shanghai Eastern Hepatobiliary Surgery Hospital were enrolled in this study. None of patients received chemotherapy or radiotherapy before surgery. Fresh liver tissues were harvested base on the ethics committee approval in Second Military Medical University (Shanghai, China). Paired peri-tumor and tumor tissues were harvested and prepared freshly, peri-tumor tissues were get from tumor tissues 1 cm far away.

Immunohistochemistry

Following antibodies: Anti-α SMA antibody (abcam), anti-interleukin 6 antibody (abcam), anti-HGF antibody (abcam), anti-SCGF antibody (R&D), anti-EpCAM antibody (abcam), anti-rabbit IgG H&L (HRP) antibody, Alexa488-conjugated goat anti-rabbit IgG antibody and Alexa568-conjugated goat anti-mouse IgG (Invitrogen) antibody, were used for this study. In negative controls, phosphate buffered saline (PBS) was used. The methods and materials for immunohistochemistry were performed as previously described [21–25]. Images of the sections were obtained using the Image-Pro Plus 4.5 software (Media Cybernetics, Silver. Spring, USA).

Cells culture and conditioned medium harvest from primary cell culture

Human hepatocellular carcinoma cell line-SMMC-7721 cells and Hep G2 cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The culture methods for these two cells were described in previously [21].

Fresh human tumor and peri-tumor tissues were cut into small pieces and put on to 6 well cell culture plates, fibroblasts were start migrate out from the each small piece of tissues from 3 to 7 days later. After 2 weeks, the small piece of tissues were removed and sub-cultured fibroblast like cells every 3 days, and from passage 3 to 10 were used for this study. Culture medium used Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF), 100 U/mL penicillin and 100 μg/mL streptomycin (sigma). To purify the primary cultured fibroblast-like cells, FACS sorting system was used, and anti-α SMA antibody (abcam) and goat anti-rabbit IgG (H + L)-Alex Fluor 488 antibody (Life technologies) were used. The method for getting CM was followed the manuscripts published by Fan QM and Liu Y before [25,26], collected and centrifuged after culture 24 h using serum-free medium.

Wound healing and matrigel invasion assay

The Wound healing and the Matrigel invasion assay followed by method that had been described previously [27], with minor modifications. For Wound healing assay, 1×10^5 SMMC-7721 cells and Hep G2 cells were seeded into 6-well culture dish and treated with CM-TAFs and CM-pTAFs for 48 h. The inhibitor and antagonists were 1 h pretreated with CM. For Matrigel invasion assay, 2×10^4 SMMC-7721 cells and Hep G2 cells were seeded on Matrigel-coated inserts, Serum free medium was added into upper space of inserts, and CM-TAFs and CM-pTAFs were added to under space of the inserts for 48 h. The inhibitor and antagonists was 1 h pretreated to the cells, and the neutralizing antibodies were 1 h pre-incubated with CM. The cells migrated and invaded under the membranes were stained with 0.1% crystal violet solution. Photographs were taken at 48 h in a phase-contrast microscope. Experiments were performed in triplicate, and four fields of each point were recorded. CCR2 antagonist RS 504393 (Apex Bio) and CXCR1/2 antagonist SCH 527123 (Apex Bio) were used in concentrations at 100 nM and 50 nM respectively. The numbers of cells invaded and migrated were counted manually.

Xenograft animal model

Six to 8 weeks old male BALB/c nude mice were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). The mice were housed under specific pathogen-free conditions. All procedures were performed in accordance with the institutional animal welfare guidelines of the Second Military Medical University. The SMMC-7721 cells (1×10^6) were injected to mice intrasplenic, and the mice were sacrificed after 1 month (The 1×10^6 Hep G2 cells were also treated same process as SMMC-7721 cells). The number of tumors on the liver surface were counted manually. Blood sampling in mice eyes were done for ALT and AST detection. The serum levels of ALT and AST were examined by a Fuji DRICHEM 55500 V (Fuji Medical System, Tokyo, Japan) according to the manufacturer's instructions. Four mice were used for each group. N = 5.

Bio-Plex Pro™ cytokine, chemokine and growth factors assay

Human cytokines, chemokines, and growth factors assay were followed by instruction manual (Bio-Rad, 10014905). CM harvested from purified TAFs and pTAFs, and used.

Flow cytometric analysis

SMMC-7721 cells were treated with CM-TAFs or CM-pTAFs for 24 h. Harvested cells were suspended in PBS and formaldehyde was added to a final concentration of 4%. The cells were fixed for 10 min at 37 °C and then chilled on ice for 1 min. Permeabilized in 90% of ice-cold methanol and then incubated for 30 min on ice. Permeabilized cells (1×10^5) were mixed with 100 μL 0.5% bovine serum albumin (BSA)/PBS containing APC anti-human CD192 antibody, FITC anti-human CD181 antibody (BioLegend), PE anti-human CD182 antibody (BioLegend) and PE anti-mouse CD326 (eBioscience) antibody for each 1.5 mL experimental tube, incubated for 1 h at room temperature. APC mouse IgG2a antibody, FITC Mouse IgG2b antibody, PE Mouse IgG1 antibody (BioLegend) were used as control. Cells were then washed with 0.1% BSA/PBS, then suspended in 0.5 mL PBS and analyzed by flow cytometry.

Western blot analysis

The 6 paired fresh HCC patients tissues were used in this study. Total protein extraction and western blot analysis were performed as described previously [21,28,29]. Anti-GAPDH antibody (Bioworld Technology), anti-NANOG antibody (Cell Signaling Technology), anti-VIMENTIN antibody (abcam) was use for this study. The Quantity One software used for analysis the related activities of each expression.

Colony formation/Suspended sphere formation assay

Colony formation assay and Suspended sphere formation assay were done parallel followed by previous publication with little modification [29,30]. The 1×10^3 CM-pTAFs or CM-TAFs treated SMMC-7721 cells were seeded into a 6-well culture dish for colony formation assay. After incubated at 37 °C for two weeks, the cells were stained with 0.1% crystal violet solution. And also, same number of cells seeded in to a low adhesive 6-well culture dish for suspended sphere formation assay. After two weeks, the number of colonies and spheres counted under the microscope. All experiments were performed in triplicate.

Cell viability

EpCAM+ SMMC-7721 cells were isolated by flow cytometric analysis, and 2×10^4 cells/well seeded to 96-well plates. 100 ng/mL of anti-HGF neutralizing antibody (abcam) was 1 h pre-incubated with/without CM-pTAFs, and treated to cells in 96- well plates. After incubated at 37 °C for 24 h, cell counting kit 8 (CCK-8) assay was done followed by instructions.

Statistical analysis

Statistical analysis was performed by using Student's *t*-test (two-tailed). The criterion for statistical significance was taken as $p < 0.05$. The results represent the mean ± SEM of three independent experiments at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control group.

Results

Propose the relativity of EpCAM+ CSCs and fibroblasts

Firstly, we observed the location of EpCAM+ CSCs in peri-tumor tissues. The results demonstrated that EpCAM+ stem cells were located close to fibroblasts in the peri-tumor tissues (Fig. 1A). Then we identified the fibroblasts in patient's tissues by immunostaining with the biomarker of active fibroblast α-smooth muscle actin (α-SMA) in tumor and peri-tumor tissues. As shown in Fig. 1B, the expression of α-SMA was positive not only in tumor tissues but also in peri-tumor tissues. Then we determined primary cultured fibroblasts captured by bright field microscopy. After 2 weeks from we put the small pieces of fresh patient's tissues into the 6-well plates, the fibroblasts migrated from the small pieces of tissues (Fig. 1C). The cells isolated from tumor and peri-tumor tissues was similar and then we did subculture to passages 10 (Fig. 1D). These