Original Articles

Ovarian cancer stem-like cells differentiate into endothelial cells and participate in tumor angiogenesis through autocrine CCL5 signaling

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ABSTRACT

Cancer stem cells (CSCs) are well known for their self-regeneration and tumorigenesis potential. In addition, the multi-differentiation potential of CSCs has become a popular issue and continues to attract increased research attention. Recent studies demonstrated that CSCs are able to differentiate into functional endothelial cells and participate in tumor angiogenesis. In this study, we found that ovarian cancer stem-like cells (CSLCs) activate the NF-κB and STAT3 signal pathways through autocrine CCL5 signaling and mediate their own differentiation into endothelial cells (ECs). Our data demonstrate that CSLCs differentiate into ECs morphologically and functionally. Anti-CCL5 antibodies and CCL5-shRNA lead to markedly inhibit EC differentiation and the tube formation of CSLCs, both in vitro and in vivo. Recombinant human-CCL5 significantly promotes ovarian CSLCs that differentiate into ECs and form microtube network. The CCL5-mediated EC differentiation of CSLCs depends on binding to receptors, such as CCR1, CCR3, and CCR5. The results demonstrated that CCL5-CCR1/CCR3/CCR5 activates the NF-κB and STAT3 signal pathways, subsequently mediating the differentiation of CSLCs into ECs. Therefore, this study was conducted based on the theory that CSCs improve tumor angiogenesis and provides a novel strategy for anti-angiogenesis in ovarian cancer.

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Introduction

Ovarian carcinoma is one of the most lethal malignancies of the female reproductive system, and statistically, 70%~80% of patients with ovarian cancer have already experienced metastasis at the time of initial diagnosis. As is well known, ovarian carcinoma is accompanied by dramatic neovascularization [1]. Many studies have reported that angiogenesis is considered a hallmark of ovarian cancer because it is indispensable to fueling tumor and leads to malignancy development, invasion, and metastasis [2]. As a new treatment strategy, anti-angiogenesis is showing promise in clinical ovarian cancer therapy [3]. However, monoclonal anti-vascular endothelial growth factor (VEGF) antibodies do not gain survival time and

exert toxic effects, such as hypertension, proteinuria, cardiac toxicity, vascular thromboembolism, hemorrhage, gastrointestinal toxicity, dermatological toxicity, and endocrine toxicities [4,5]. Therefore, exploring the underlying mechanism of angiogenesis in ovarian carcinoma would benefit the understanding of the mechanisms of tumor occurrences and development and provide a new target for anti-angiogenesis clinical tumor treatment.

Tumor angiogenesis involves degradation of the extracellular matrix, endothelial cell (EC) migration, proliferation, elongation, and tube formation to form new vessels [6]. Pre-existing ECs, bone marrow-derived endothelial progenitor cells (EPCs) and mesenchymal stem cells have traditionally been regarded as the sources of ECs in tumor angiogenesis [7,8]. However, recent studies indicate that cancer stem cells (CSCs) play a marked role in tumor angiogenesis: First, CSCs promote EC-mediated angiogenesis through the recruitment of EPCs toward tumor tissues via the production of angiogenin-1 and angiogenin-2 [9], as well the enhancement of the proliferation and differentiation of ECs via the secretion of proangiogenic factors, such as VEGF [10,11]. Second, VEGF produced by CSCs contribute to the formation of mimic vessels that provide nutrition and oxygen to tumor tissues in the absence of ECs [12–14]. Third, CSCs possess stem cell characteristics that make them capable of differentiating into ECs. It has been demonstrated that

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CSCs differentiate into functional vascular ECs in glioblastoma [15,16] and breast cancer [17]. Furthermore, in both glioblastoma and breast cancer, the differentiation of CSCs depends on autocrine and paracrine VEGF production [16,17]. However, whether other factors also contribute to this EC differentiation and whether CSCs can differentiate into ECs and subsequently tumor angiogenesis in other tumors, such as ovarian cancer, remain unanswered questions.

Consequently, our previous studies demonstrated that ovarian CSLCs abundantly self-produce the chemokine CCL5 and its receptors, and that CCL5 signaling plays a critical role in the migration and invasion of ovarian cancer [18]. Whether autocrine CCL5 signaling mediates other biological behaviors in ovarian CSLCs, such as angiogenesis, remains unknown. Moreover, it has been reported that the chemokine CCL5 via promoting VEGF expression-mediated EPCs-derived angiogenesis in human chondrosarcoma [19–21], in addition, promotes angiogenic phenotype of glioblastoma [22] and breast cancer [23], indicating that CCL5 is associated with tumor angiogenesis. However, the relevance of chemokine CCL5 in the endothelial differentiation of ovarian CSLCs has not been investigated.

In this study, we found that ovarian CSLCs differentiate into ECs both morphologically and functionally. Further study uncovered a novel mechanism underlying the differentiation of CSLC into ECs, at least in ovarian cancer, that differs from whatwas previously reported.

Materials and methods

Cell culture

The ovarian cancer cell line (A2780 cell line) and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC). These cell lines were tested and authenticated by short tandem repeat profiling in August 2015. A2780 cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin. HUVECs were maintained in EBM medium (Cambrex). Ovarian CSLCs and non-CSLCs (NCSLCs) were generated from the A2780 cell line according to a previously published protocol [18,24–26]. Briefly, the isolated CSLCs were cultured in low-attachment plates under stem cell conditions consisting of serum-free medium supplemented with 5 μg/mL insulin (Sigma), 20 ng/mL recombinant epidermal growth factor (Invitrogen), and 10 ng/mL basic fibroblast growth factor (Invitrogen), as previously described. Ovarian CSLCs were characterized by flow cytometry with antibodies against CD133 (293C3-allophycocyanin; Miltenyi Biotech). NCSLCs derived from the A2780 cell line were characterized as CD133-negative cells through flow cytometry. All of the cells were maintained in an incubator at 37 °C with 5% CO_2.

Tube formation assay

Matrigel (BD Biosciences) was dissolved at 4 °C overnight; μ-slides for angiogenesis (ibidi) or 96-well plates were prepared at 4 °C for 60 min. Ten microliters (for μ-slides) or thirty microliters (for 96-well plates) of Matrigel was placed in each well, and the plates were incubated at 37 °C for 30 min [27–29]. Cells (1×10^5/200 μL) in the basic endothelial medium with or without recombinant human CCL5 (rh-CCL5, 30 ng/ml, R&D Systems) were added to the Matrigel-treated wells. For antibody inhibition experiments, ovarian CSLCs were incubated with either monoclonal anti-CCL5 antibodies (R&D Systems) at concentrations of 50, 250, and 500 ng/mL, or an anti-CCR1/3/5 antibody (BD Biosciences) at 10 μg/ml. For chemical inhibition experiments, ammonium pyrrolidine dithiocarbamate (PDTC; Beyotime Biotechnology) at a concentration of 0.5 μmol/L and cucurbitacin (Gene Operation) at different concentrations of 0.0025, 0.005, 0.01, and 0.02 nmol/L were added to the system. All of the formed tubes and networks were imaged by inverted phase-contrast microscopy. The tube branch and total tube lengths were calculated using the MacBiophotonics Image J software.

In vitro cell proliferation assay

Cells were cultured in 96-well plates. Cell growth was measured every 6 h using a cell counting kit-8 (CCK8, Beyotime). Cells (3×10^3, 100 μL) were seeded in the presence of PDTC at a concentration of 0.5 μmol/L or of cucurbitacin (Gene Operation) at different concentrations of 0.0025, 0.005, 0.01, and 0.02 nmol/L and incubated for 4 h at 37 °C with 5% CO2. After 10 μL, WST-8 dye was added to each well, cells were incubated at 37 °C for 2 h and the absorbance was detected at 450 nm wave length using a microplate reader; each treatment was done in triplicate.

Immunofluorescence

The immunofluorescence analysis was performed on 6-μm frozen tissue sections, cell-paved μ-slides, or spheroids of cells centrifuged on normal glass slides. The above-mentioned sections were fixed with ice-cold 4% paraformaldehyde for 20 min at 37 °C, blocked with normal serum for 20 min at room temperature and then incubated with specific antibodies against mouse monoclonal CD31 (1:200, rabbit anti-mouse, Cell Signaling Technology), human monoclonal CD31 (1:200, rabbit anti-human, Cell Signaling Technology), VE-Cad (1:200, rabbit anti-human, Abcam), Von Willebrand factor (vWF) (1:200, rabbit anti-human, Cell Signaling Technology), NF-κB (1:500, rabbit anti-human, Cell Signaling Technology), or STAT3 (1:500, rabbit anti-human, Cell Signaling Technology) overnight in the dark at 4 °C. After three washes, the slides were stained with Cy3-conjugated anti-mouse antibodies or anti-rabbit antibodies (1:200, Abcam). The nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI), and the stained sections were then visualized with an Olympus confocal microscope. All immunofluorescence photomicrographs were analyzed using Image Pro Plus (IPP, version 6.0). The number of positive cells per field of view under 1200× or 400× magnification was counted to determine the positive index, which was calculated for five randomly selected views by two pathologists and analyzed using the GraphPad software.

Immunohistochemistry

For immunohistochemistry staining, mouse xenograft tissues were cut into 5-μm thick serial sections. The specimens were then incubated with antibodies against mouse CD31 (1:50, rabbit anti-mouse, Cell Signaling Technology) or human CD31 (1:50, mouse anti-human, Cell Signaling Technology). The secondary antibody, i.e., an anti-rabbit or anti-mouse peroxidase-labeled polymer (1:50, ZSGB Bio), was linked to the primary antibody for 30 min. All immunohistochemical photomicrographs were analyzed using Image Pro Plus (IPP, version 6.0). The number of positive cells per field of view under 400× magnification was counted to determine the positive index, which was calculated for five randomly selected views by two pathologists and analyzed using the GraphPad software.

Western blotting

After cell collection, a nuclear and cytoplasmic protein extraction kit (KeyGen Biotech) was used, and the extracts were resolved by SDS-polyacrylamide gel electrophoresis using PVDF transfer membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies (1:1000, Cell Signaling Technology) against p65, p-p65, STAT3 and p-STAT3 overnight at 4 °C. After three washes, the blots were incubated with mouse anti-rabbit peroxidase-conjugated secondary antibody (1:5000, Beyotime Biotechnology) for 1 h at room temperature, washed three times, and visualized by enhanced chemoluminescence. Each experiment was repeated at least three times.

RNA interference

CCL5 short hairpin RNA (shRNA) oligonucleotides were obtained from a commercial source (Neuron Bio). Briefly, a stem-loop structure oligonucleotide containing a CCL5-target sequence (5'-GTGTGTGCCAACCCAGAGA-3') was cloned under the control of the human U6 promoter into lentiviral vectors that also contained a green fluorescent protein (GFP) reporter [18]. The control shRNA incorporated no inserted loop structure. The day before transduction, the cells were dissociated and transduced with shRNA in accordance with the manufacturer's protocol. After 48 hours, the medium was replaced, and the cells were harvested for additional experiments. The knockdown efficiency of CCL5-shRNA was determined by RT-PCR and Western blot analyses.

In vivo xenograft experiments

Nude mice were purchased from the Chinese Academy of Medical Sciences. The mice (female, 4–5 weeks of age) were housed and maintained in laminar flow cabinets under specific, pathogen-free conditions. For the xenograft experiments, NCSLCs (1×10^6), CSLCs (1×10^6) and CSLCs (1×10^6) infected with lentivirus carrying GFP-shRNA or CCL5-shRNA were injected subcutaneously into the right flank of the recipient mice (n = 6 mice/group). To determine the size of the subcutaneous tumors, the greatest longitudinal diameter (A) and the greatest transverse diameter (B) were estimated with external calipers, and the tumor volume was calculated as $1/2(A \times B^2)$ mm^3. The mice were killed once the xenograft tumor reached a size of 500–600 mm^3 at different days, and the tumor xenografts were harvested for further evaluation by immunofluorescence or immunohistochemistry. The mice were cared for and used in accordance with The Third Military Medical University ethical guidelines.

Statistical analysis

All of the data from the quantitative assays are expressed as the means ± SD. Statistical analyses were performed using independent-sample t-tests or one-way ANOVA. The following symbols indicate that the samples presented statistically sig-