Epithelial-mesenchymal transition contributes to malignant phenotypes of circulating tumor cells derived from gastric cancer

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Abstract: Circulating tumor cells (CTCs) are crucial to tumor metastasis, and they usually undergo epithelial–mesenchymal transition (EMT) in order to disseminate from the primary tumor. However, very little is currently known about the relationship between EMT and malignant phenotypes of CTCs in the context of gastric cancer. Therefore, this study aimed to investigate the contribution of EMT to malignant phenotypes of CTCs derived from gastric cancer cells. We xenografted MKN28 gastric cancer cells pretreated with transforming growth factor-beta 1 (TGFβ-1) into nude mice by intravenous injection. Next, we isolated CTCs from the blood of nude mice by gradient centrifugation and found that CTCs derived from MKN28 cells pretreated with TGFβ-1 had a significantly increased viability and invasion ability compared to MKN28 cells without TGFβ-1 treatment. Immunocytochemical staining showed lower expression of E-cadherin and higher expression of N-cadherin, vimentin, and β-catenin in CTCs derived from MKN28 cells pretreated with TGFβ-1. Furthermore, the expression of Wnt3a, β-catenin, cyclin D1, and c-Myc was significantly higher in CTCs derived from MKN28 cells pretreated with TGFβ-1. Taken together, these findings suggest that TGFβ promotes EMT and malignant phenotypes of gastric cancer cells. Furthermore, the malignant phenotypes of gastric cancer cells induced by TGFβ are maintained in CTCs derived from these cells. Targeting EMT in CTCs is a new approach to the treatment of gastric cancer relapse and metastasis.

Introduction

Gastric cancer (GC) is one of the most common malignant human cancer and causes significant cancer-related death worldwide (Lei et al., 2017). Most GC patients are diagnosed at a late stage, and their poor prognosis is mainly due to the dissemination of tumor cells into the circulation as circulating tumor cells (CTCs) and consequent formation of distal tumor by CTCs, especially in the liver (Kakeji et al., 2010; Yang et al., 2018). CTCs are crucial to tumor metastasis, and they usually undergo epithelial-mesenchymal transition (EMT) in order to disseminate from the primary tumor (Fabisiewicz and Grzybowska, 2017; Zhang et al., 2018).

EMT is a key event during tumor invasion in which epithelial cells acquire mesenchymal characteristics and lose cell polarity (Chen et al., 2018). EMT is a common mechanism for distant dissemination, accounting for cancer-related mortality. Recent studies in a variety of solid cancers have shown that CTCs undergo EMT to drive tumor progression, and EMT phenotypes of CTCs could predict poor prognosis of cancer patients (Guan et al., 2019; Zhao et al., 2019). In particular, in non-small cell lung cancer (NSCLC) patients, EMT markers were expressed at significantly higher proportions in CTCs than patient-matched NSCLC tissues, and EMT positive CTCs were associated with significantly poor survival of NSCLC patients (Manjunath et al., 2019). In contrast, the inhibition of EMT in CTCs led to reduced cancer metastasis (Mendonça et al., 2019). These results indicate that the EMT of CTCs plays an important role in tumor metastasis.

Unfortunately, very little is currently known about the relationship between EMT and malignant phenotypes of CTCs in the context of GC. Therefore, in this study, we aimed to investigate the contribution of EMT to malignant phenotypes of CTC derived from GC.

Materials and Methods

Cell culture

Human gastric cancer cell line MKN28 was purchased from
American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL). Transforming growth factor-beta 1 (TGFβ-1) was purchased from Sigma (St. Louis, MO, USA). MKN28 cells were treated with TGFβ-1 at 10 ng/mL for 48 h.

**Animal model**

Balb/c nude mice (5-week-old) were provided by the Animal Center of Kunming Medical University, and all animal experiments were approved by the Animal Use and Care Committee of Kunming Medical University. The untreated or TGFβ-1 treated MKN28 cells (1 × 10^6 cells per mouse) were inoculated intravenously into Balb/c nude mice. The growth of tumor cells was monitored, and the mice were sacrificed after 10 weeks. The liver tissues were dissected, fixed in 10% neutral-buffered formalin, embedded, and cut into sections for hematoxylin-eosin staining following routine procedures.

**Isolation and identification of CTCs**

CTCs were isolated from mice inoculated with MKN28 cells using the method described previously (Zhang et al., 2016). Briefly, 0.7 mL blood was collected from the orbital sinus of the mice. Mononuclear cells were collected and subjected to gradient centrifugation to isolate CTCs. The isolated CTCs were characterized by immunofluorescence assay and flow cytometry.

**Invasion assay**

Invasion assay was performed using Transwell inserts (Corning) following the manufacturer's protocol. MKN28 cells or CTCs were diluted in serum-free medium and loaded into the upper side of inserts coated with Matrigel. The underside of the insert was loaded with 10% FBS. After incubation at 37°C for 48 h, the inserts were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) and then stained with 2% crystal violet. The cells that had invaded the underside were counted in five randomly selected fields per insert.

**Cell viability assay**

MKN28 cells or CTCs were seeded in 96-well plates at 1 × 10^4 cells/well. The cells were cultured in a humidified incubator at 37°C. Three days later, viable cells were detected with a CCK-8 assay kit (Dojindo Lab, Kumamoto, Japan) following the manufacturer’s protocol. The optical density was read at 450 nm using a microplate reader.

**Immunofluorescence assay**

MKN28 cells or CTCs were fixed in 3% formaldehyde, permeated with 0.1% Triton X-100, blocked in 3% bovine serum albumin (BSA) in PBS, and then sequentially incubated with primary antibodies against E-cadherin, N-cadherin, vimentin and β-catenin (Abcam) overnight at 4°C, and fluorescent-labeled secondary antibody (Jackson) in the dark. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma), and the cells were observed under a fluorescence microscope (Leica, Mannheim, Germany).

**FIGURE 1. Characterization of CTCs isolated from mice with tumor xenograft.** MKN28 cells pretreated with or without TGFβ-1 were xenografted into nude mice by intravenous injection. CTCs were isolated from the blood by gradient centrifugation. (A) Immunofluorescence assay of CTCs by CK19 antibody (Green). The nuclei were stained as blue. Magnification: 100X. (B) Flow cytometry analysis of CK19 (orange) and CD45 (green). M28: MKN28 cells untreated. E28: MKN28 cells pretreated with TGFβ-1.
Real-time PCR
Real-time PCR was performed as described previously (Zhong et al., 2018). Briefly, total RNA was extracted from cells, and cDNA was produced by reverse transcription. PCR amplification of β-catenin, Wnt1, Wnt3, Wnt3a, cyclinD1, c-Myc, and GAPDH was performed using SYBR Green Master Mix (PE Biosystems) on ABI Prism 7000 Sequence Detector (Applied Biosystems). Relative mRNA levels were normalized to GAPDH and calculated using the Ct method.

Statistical analysis
Data were expressed as the mean ± standard derivation (SD) and analyzed using SPSS 12 statistical analysis package (SPSS Inc., Chicago, IL, USA). The comparison between the two groups was made by a t-test, and p < 0.05 was regarded as significant.

Results
Isolation and characterization of CTCs from mice with tumor xenograft
First, we xenografted MKN28 cells pretreated with or without TGFβ-1 into nude mice by intravenous injection. Next, we isolated CTCs from the blood of nude mice by gradient centrifugation and then performed CK19 immunofluorescence assay because CK19 is an epithelial cytoskeleton marker. The results showed that CTCs were positively stained by the CK19 antibody, indicating that they were derived from epithelial cells (Fig. 1(A)). Furthermore, flow cytometry analysis showed that CTCs were positive for CK19 but negative for CD45, a marker of hematopoietic cells (Fig. 1(B)). These data confirmed that the isolated CTCs were derived from xenografted MKN28 cells and not from hematopoietic cells.

CTCs showed increased viability and morphological changes
CCK-8 assay showed that MKN28 cells pretreated with TGFβ-1 (E28 cells) had significantly increased viability compared to cells without TGFβ-1 treatment (M28 cells). In addition, CTCs derived from MKN28 cells xenografted in mice showed increased viability. In particular, CTCs derived from E28 cells (E28-CTC) had significantly increased viability compared to cells without TGFβ-1 treatment (M28-CTC) (Fig. 2(A)). For cell morphology, M28 or M28-CTC cells were polygonal with short protrusions and had no pseudopods. In contrast, E28 or E28-CTC cells had pseudopods and loose cell gap (Fig. 2(B)).

CTCs showed increased invasion ability
Transwell assay showed that more cells of E28 or E28-CTC group invaded the membrane than M28 or M28-CTC group (Fig. 3(A)). Statistical analysis showed that the difference in invasion ability between E28 or E28-CTC and M28 or M28-CTC was significant (p < 0.05, Fig. 3(B)).
FIGURE 3. CTCs showed increased invasion. (A) Transwell assay of cell invasion. (B) The number of invaded cells. M28: MKN28 cells untreated. E28: MKN28 cells pretreated with TGFβ-1. M28-CTC: CTC derived from M28. E28-CTC: CTC derived from E28. *p < 0.05 (N = 3).

FIGURE 4. Immunocytochemical staining of EMT markers in gastric cancer cells and CTCs. (A–D) E-cadherin, N-cadherin, vimentin, and β-catenin staining. (E) Quantitative analysis of staining intensity. M28: MKN28 cells untreated. E28: MKN28 cells pretreated with TGFβ-1. M28-CTC: CTC derived from M28. E28-CTC: CTC derived from E28.
CTCs underwent EMT
To understand why CTCs had increased viability and invasion, we examined EMT markers. Immunocytochemical staining showed that the expression of E-cadherin was lower while the expression of N-cadherin, vimentin, and β-catenin was higher in E28 or E28-CTC than in M28 or M28-CTC (Figs. 4(A)-4(E)).

The activation of Wnt/β-catenin signaling in CTCs
To elucidate the mechanism by which CTCs undergo EMT, we focused on Wnt/β-catenin signaling because it is crucially involved in EMT of tumor cells and tumor metastasis (Zheng et al., 2017). Real-time PCR analysis showed that the expression of Wnt1, Wnt3a, β-catenin, cyclin D1, and c-Myc was significantly higher in E28 cells than in M28 cells. In addition, the expression of Wnt1, Wnt3a, β-catenin, cyclin D1, and c-Myc was higher in M28-CTC than in M28 cells. However, only the expression of Wnt3a, β-catenin, cyclin D1, and c-Myc was significantly higher in E28-CTC than in M28-CTC cells (Fig. (5)).

Discussion
CTCs have been regarded as a new diagnostic marker of malignant tumors because they are shed from the primary tumor mass and then circulate in the peripheral blood, which allows for easy and sensitive detection (Lyu et al., 2019). In particular, the presence of CTCs in the peripheral blood could predict poor prognosis of patients with gastric cancer (Qiu et al., 2010; Kubisch et al., 2015; Xia et al., 2015; Yang et al., 2018). However, the detailed mechanism by which CTCs contribute to the poor prognosis of gastric cancer patients remains largely unclear.

Invasion and metastasis are crucial to the progression of cancer and are often the cause of cancer-related death. EMT is originally a vital process in embryo development in which adherent epithelial cells gain the capability to migrate, in order to provide support for intense cell movement during embryogenesis (Wang and Steinbeisser, 2009). Unfortunately, tumor cells undergo EMT to regain the capability to migrate and increase the aggressiveness. Furthermore, EMT is responsible for the drug resistance of tumor cells to drive cancer recurrence and metastasis (Wang, 2010). Emerging evidence suggests that CTCs undergo EMT to disseminate from the primary tumor, circulate in the blood and then metastasize into distinct sites, leading to the poor outcome of cancer patients (Mego et al., 2019).

TGFβ is considered as a major regulator of cancer metastasis, promoting EMT-associated cell migration and invasion (Ungefroren et al., 2018). Therefore, in this study, we treated gastric cancer cells with TGFβ-1. The cells treated with TGFβ-1 showed increased viability and invasion, and they exhibited EMT phenotypes, including loose cell gap, the existence of pseudopods, and changes in expression of EMT markers. In detail, the expression of the epithelial marker E-cadherin decreased while the expression of the mesenchymal markers N-cadherin and vimentin increased in gastric cancer cells after treatment with TGFβ-1. Moreover, gastric cancer cell-derived CTCs xenografted into mice maintained the similar phenotypes of EMT.

Wnt signaling is known to promote cancer cell invasion (Tian et al., 2017). Therefore, we examined Wnt/β-catenin signaling in gastric cancer cells and CTCs. We found that the expression of Wnt3a, β-catenin, cyclin D1, and c-Myc was significantly higher in CTCs derived from gastric cancer cells treated with TGF-β. Since cyclin D1 and c-Myc are important downstream effectors of Wnt/β-catenin signaling, our data suggest that canonical Wnt/β-catenin signaling is responsible for EMT of CTCs derived from gastric cancer cells. However, it is proposed that non-canonical Wnt/PCP signaling plays an important role in cancer cell invasion and metastasis (Wang, 2009). Further studies are needed to dissect the contribution of different branches of Wnt signaling to EMT of CTCs.

In summary, our results demonstrate that TGFβ promotes EMT and malignant phenotypes of gastric cancer cells. Furthermore, the malignant phenotypes of gastric cancer cells induced by TGFβ are maintained in CTCs derived from these cells. Targeting EMT in CTCs is a new approach to the treatment of gastric cancer relapse and metastasis.

Conflict of Interest
The authors declare no conflict of interest.
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