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ORIGINAL ARTICLE

Epidermal growth factor enhances chemosensitivity of colon cancer by inducing cancer stem cells to enter the cell cycle

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Abstract

Objective The aim of the study was to investigate whether colon cancer stem cells induced by epidermal growth factor (EGF) to enter the cell cycle enhanced the chemosensitivity of colon cancer.

Methods *In vitro*, EGF was used to stimulate the entry of human colon cancer HCT116 cells into the cell cycle. Before and after treatment with EGF, CD133+ HCT116 cells were collected and flow cytometry was conducted to determine the apoptosis rate based on the 5-Fu and Ki-67 expression rates. The cell cycle distribution of the two groups was also determined. *In vivo*, a subcutaneous xenograft model of HCT116 human colon cancer cell lines in nude mice was established. The nude mice were divided into two groups and treated with EGF and 5-Fu, respectively. Differences in the growth of implanted tumors revealed the efficiency of cycle-induction combined chemotherapy.

Results (1) After EGF stimulation, the S-G2/M proportion of CD133+ HCT116 cells and Ki67 expression were increased, indicating that more cancer stem cells entered the cell cycle and promoted proliferation; (2) After EGF stimulation, CD133+ HCT116 cells showed a higher apoptosis rate induced by 5-Fu. (3) Animal experiments showed that the group subjected to combined treatment with EGF and 5-Fu had smaller tumor sizes compared to the group treated with 5-Fu alone.

Conclusion EGF enhanced tumor sensitivity to chemotherapeutic drugs, likely by promoting tumor stem cells to enter the cell cycle.

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Key words: chemosensitivity; tumor stem cell; cell cycle

Colorectal cancer is a common malignant tumor with high mortality. Solid tumors are composed of heterogeneous cell populations with different biological characteristics. A small number of cells known as cancer stem cells (CSCs) can sustain the malignant population [1–3], and the population was found to be correlated with multidrug resistance, tumor recurrence, and low patient survival [4–8]. Recent studies showed that in leukemia and in solid tumors, CSCs are particularly resistant to conventional chemo and radiation therapies compared to more differentiated cells in the non-CSC compartment, which comprise the tumor bulk. It was reported that CD34+/CD38– leukemia precursors cells have reduced sensitivity to daunorubicin, a major drug used in leukemia treatment, compared to leukemic blasts (CD34+/CD38+ counterpart) [9–10]. Furthermore, CSCs from acute and chronic myelogenous leukemia are relatively quiescent and contribute to chemotherapy resistance because the sensitivity to chemotherapeutic agents relies upon lethal cellular damage during cell cycle progression in highly proliferative cells [11–12]. Ishikawa *et al* [13] demonstrated that the bone marrow endosteal region was enriched in quiescent human acute myeloid leukemia (AML) stem cells (LSCs) and *in vivo* cytokine treatment induced their entry into the cell cycle. Using an *in vivo* treatment strategy in an NOD/SCID/IL2rγnull human AML xenotransplantation model, elimination of human primary AML LSCs was significantly enhanced through *in vivo* cell cycle modification [13]. However, it was not reported whether this strategy is effective for solid tumors. In our study, by using epidermal growth factor (EGF) to induce the entry of colon cancer cells into

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the cell cycle and using CD133 as a marker of colorectal CSCs, we found that EGF enhanced tumor sensitivity to chemotherapeutic drugs, likely by promoting tumor stem cells to enter the cell cycle.

Materials and methods

Cell culture

The human colon cancer cell line HCT116 was obtained from the China Centre for Type Culture Collection (CCTCC; Wuhan, China) and maintained in RPMI-1640 (Hyclone, Logan, UT, USA) medium supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Chemical treatment

HCT116 cells were seeded at 1×10^6 cells per well in 6-well culture plates. After 24 h, the culture medium was changed to contain medium free of bovine serum. EGF and 5-fluorouracil (5-FU) were added to the cell culture at concentrations shown in Table 1. The cells were harvested after 24 h and 48 h and prepared for analysis.

CD133 expression analysis by flow cytometry and cell sorting

CD133 expression was evaluated by direct immunofluorescent staining using the AC133 mouse monoclonal antibody (anti-CD133-PE, AC133, Miltenyi Biotec, Bergisch Gladbach, Germany) directly conjugated with phycoerythrin. Mouse IgG1-phycoerythrin was used as the isotype control antibody. All cells were stained according to the manufacturer's recommendations. Briefly, 2×10^5 live cells were suspended in 100 μL of buffer (0.5% fetal calf serum and 2 mM EDTA) and stained for 10 min at 4 °C with 10 μL of the AC133 antibody (1:11). Cells were washed to remove excess unbound antibodies and analyzed for phycoerythrin expression by flow cytometry on a FACSort cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Ten thousand events were acquired and analyzed using Cellquest (BD Biosciences) software. To sort cells expressing CD133, the cells were stained as described above and resuspended in the same buffer at 10^6 cells/mL. Sorting was performed on a FACSVantage flow cytometer (BD Biosciences). The CD133+ cells were collected.

Ki-67/DNA multiparameter assay

Harvested cells were fixed in ice-cold 80% ethanol at –20°C for at least 24 h. Next, they were washed in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS for 5 min on ice. After centrifugation, the cells were incubated overnight in the presence of primary antibody against Ki-67 (BD Pharmingen, Franklin Lakes, NJ, USA; diluted in PBS containing 1% bovine serum albumin) and then rinsed and incubated with secondary fluorescein isothiocyanate (FITC)-conjugated antibody (DAKO, Santa Clara, CA, USA; diluted in PBS containing 1% BSA) for 30 min. Cells were then re-suspended in propidium iodide solution (50 μg/mL propidium iodide, 500 μg/mL RNase) and incubated at room temperature for 30 min. Cell fluorescence was measured using a FACSort flow cytometer and Ki-67 level and DNA profile were analyzed using Cellquest software.

Table 1 Chemical treatment for different groups of HCT116 cells

Treatment	Con	EGF	5-FU	EGF + 5-FU
EGF (ng/mL)	0	25	0	25
5-FU (mg/mL)	0	0	0.025	0.025

Apoptosis assay

After different treatments, cells were harvested, washed with cold PBS, and then suspended in 100 μL cold annexin V binding buffer, 5 μL annexin V- FITC, and propidium iodide (Jingmei Company, Beijing, China). After the samples were incubated for 15 min at room temperature in the dark, the cells were analyzed on a FACSort flow cytometer. Quadrants were set based on the analysis of single-stained samples. The percentage of apoptotic cells was analyzed with Cellquest software.

Mouse xenograft model of HCT116 cells and chemical treatment

BALB/c-nu/nu 4-week-old athymic mice (nude mice) were purchased from the Slaccas Company (Shanghai, China). Each mouse was injected subcutaneously with 1×10^6 HCT116 cells. When the xenograft tumors reached 0.1 cm^3 in volume, the mice were randomized into two groups, with six mice in each group. The 5-FU group was administered by subcutaneous injection of saline, intraperitoneal injection of 5-FU at a dose of 1.25 g/kg/day and the EGF + 5-FU group was administered by subcutaneous injection of EGF at a dose of 25 μg/kg/day and intraperitoneal injection of 5-FU at a dose of 1.25 g/kg/day. Health was monitored and tumor size was measured every day using a caliper. Tumor volume was calculated as length × width × width/2.

Statistical analysis

All statistical analyses were carried out using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). All *in vitro* experiments were conducted at least three times. Data were expressed as the mean ± SD. A *P*-value of <

0.05 was considered significant.

Results

FACS-sorted CD133+ cells from EGF-treated HCT116 cells showed higher Ki-67 expression and S + G2/M proportion than FACS-sorted CD133+ cells from untreated cells

HCT116 cells were divided into two groups, a control group and EGF group (final concentration of EGF was 25 ng/mL, treated for 24 h). We observed CD133 expression in HCT116 cells (Fig. 1a). Next, the CD133+ cells were sorted by FACS. We found that CD133+ cells in the EGF treated group showed higher Ki-67 expression (Fig. 1b and 1c, $P < 0.05$) and higher S + G2/M (Fig. 1d and 1e, $P < 0.05$) than those in the control group. These results indicate that EGF stimulated the CD133+ CSCs to proliferate by inducing entry into the cell cycle.

EGF enhanced sensitivity of HCT116 cells to 5-FU *in vitro*

To determine whether EGF can change the chemotherapeutic effect of cytotoxic drugs, we designed the following experiment: four cell groups, a control group, EGF group, 5-FU group, and EGF + 5-FU group, were treated as listed in Table 1. The control group was treated with saline solution rather than EGF or 5-FU. The cells were treated for 24 h or 48 h and then harvested to analyze apoptosis. Apoptosis was analyzed by flow cytometry. After 24 h and 48 h, the apoptosis rate was determined (Fig. 2a and 2b, $P < 0.05$). These results demonstrate that EGF can increase the chemosensitivity of HCT116 cell to the cytotoxic drug 5-FU.

EGF enhanced sensitivity of HCT116 cells to 5-FU *in vivo*

We next assessed the effect of EGF on chemotherapy *in vivo* in a mouse xenograft model. The 5-FU group was administered subcutaneous injection of saline and intraperitoneal injection of 5-FU at a dose of 1.25 g/kg/day; the EGF + 5-FU group was administered subcutaneous injection of EGF at a dose of 25 μg/kg/day and intraperitoneal injection of 5-FU at a dose of 1.25 g/kg/day. Tumor size was measured every day. The growth curve showed that in the EGF + 5-FU group, the tumor grew more slowly than in the 5-FU group (Fig. 3); there was significant difference between the groups ($P < 0.05$). However, in both groups, the tumors continued growing and growth did not slow as expected.

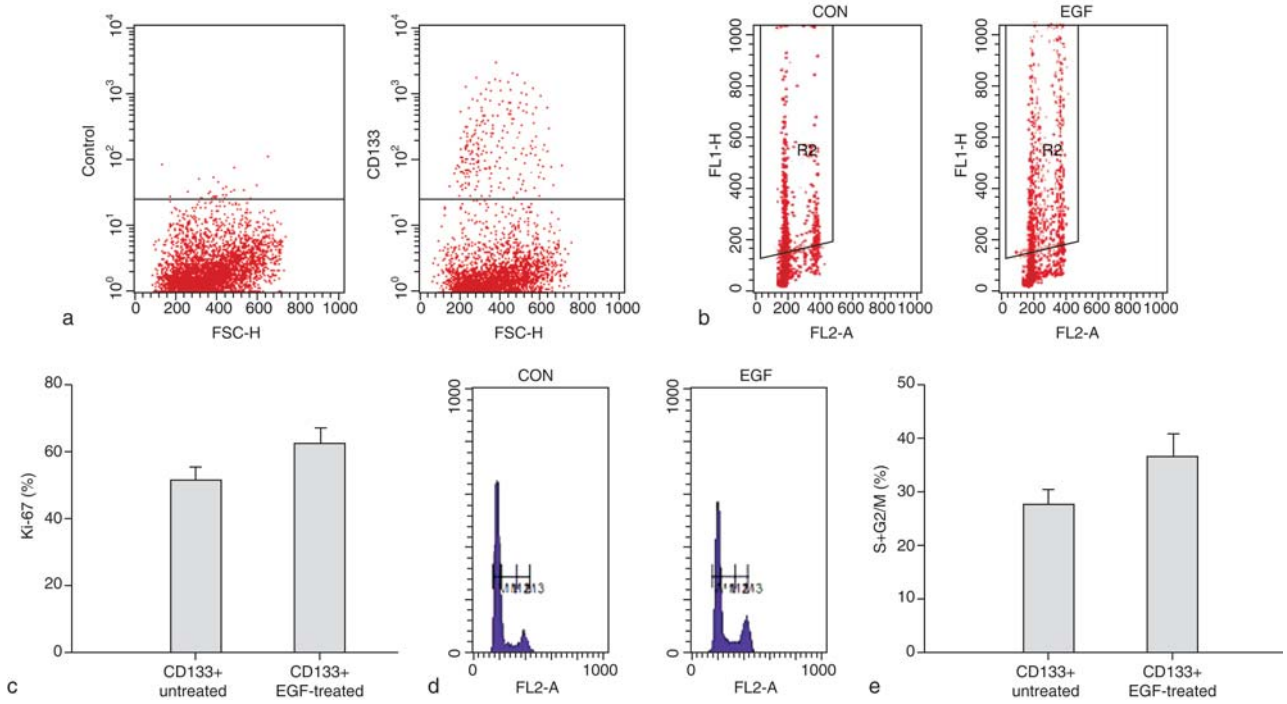

Fig. 1 Comparison of Ki-67 expression and S + G2/M proportion between FACS sorted CD133+ cells from EGF treated HCT116 cells and untreated HCT116 cells. (a) Flow cytometric analysis of CD133 expression in HCT116 cells. The left was negative control. (b and c) CD133+ cells sorted from EGF treated group showed higher Ki-67 expression than the group not treated by EGF ($P < 0.05$). (d and e) CD133+ cells sorted from EGF treated group showed higher S + G2/M proportion than the group not treated by EGF ($P < 0.05$). Data were presented as mean ± SD.

Discussion

Recurrence and metastasis are important issues in tumor treatment. The CSC theory states that CSCs have self-renewal capacity and undergo pluripotent differentiation into multiple cell types. Such cells are thought to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors [2–3]. Therefore, the development of specific therapies targeted at CSCs shows promise for improving the survival and quality of life of patients with cancer. Despite developments in molecularly targeted therapies, cytotoxic chemotherapy remains one of the mainstays of cancer treatment. Many studies have shown that CSCs are chemoresistant. Current cancer therapies are designed to target highly proliferating tumor cells. The determination of tumor shrinkage concomitant with the mean disease-free survival of patients is commonly used as an indicator of treatment efficacy. While such strategies eliminate the visible portion of the tumor, namely the tumor mass, they mostly fail to eliminate the unseen root of cancer, namely CSCs, which possess an inherent resistance towards cytotoxic compounds and radiation and thus are capable of surviving therapy [5–8]. Therefore, it is necessary to develop new strategies to eliminate CSCs [14–16]. Ishikawa *et al* [13] demonstrated that the bone marrow endosteal region is enriched for quiescent human AML LSCs and *in vivo* cytokine treatment induces their entry into the cell cycle. Using an *in vivo* treatment strategy in a NOD/SCID/IL2rγnull human AML xenotransplantation model, significantly enhanced elimination of human primary AML LSCs was achieved through *in vivo* cell cycle modification [13]. Studies of hematopoietic stem cells (HSCs) enabled evaluation of LSCs, which resemble normal HSCs in their ability to engraft, produce progeny long-term, and self-renew *in vivo* [17–19]. However, few studies have examined CSCs in solid tumors. It was recently reported that inhibition of Cdk2 kinase activity selectively targets the CD44+/CD24–/low stem-like subpopulation and restores the chemosensitivity of SUM149PT triple-negative breast cancer cells [20]. Because Cdk2 is an important kinase in cell cycle regulation, we evaluated whether changing the cell cycle status of cancer stem cells increased the chemosensitivity of the population.

Experiments confirmed that CD133 could be used as a CSC marker for prostate, lung, melanoma, and liver cancer, as well as other tumors [21–25]. O'Brien *et al* [26] isolated CD133+ cells from specimens of colorectal cancer, inoculated different concentrations of CD133+ cells into NOD/SCID mice under the renal capsule, detected tumors of CD133+ cells with the ability to self-renew and differentiate, and created heterogeneous tumor cell populations. Ricci-Vitani [27] also found enrichment of CD133+ cells in colon CSCs. In a serum-free medium, CD133+ cells, which stayed in the form of undifferentiated tumor balls for 1 year, retained tumorigenic ability, while CD133– cells did not. CD133 expression in tumor cells

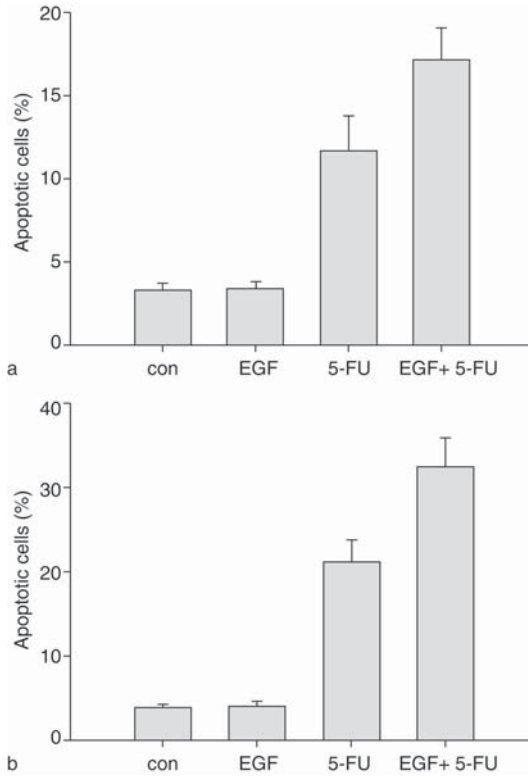

Fig. 2 Analysis of chemical treatments induced apoptosis of HCT116 cells *in vitro*. Apoptosis were detected by flowcytometry. (a) After 24 hours, apoptosis rate was 11.67% ± 2.11% in 5-FU group versus 17.15% ± 1.89% in EGF + 5-FU group ($P < 0.05$). (b) After 48 hours, apoptosis rate was 20.97% ± 2.69% in 5-FU group, versus 32.42% ± 3.36% in EGF + 5-FU group ($P < 0.05$). Data were presented as mean ± SD.

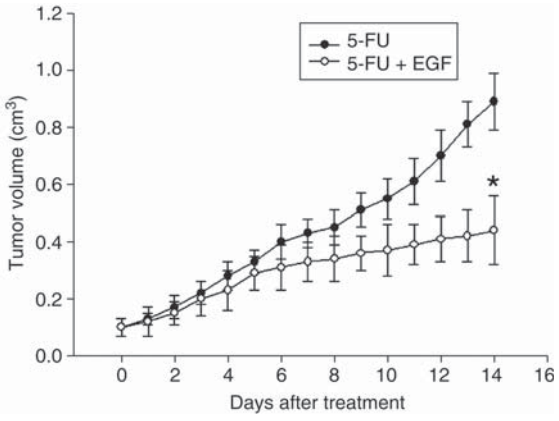

Fig. 3 EGF enhanced sensitivity of HCT116 cells to 5-FU *in vivo*. Tumor size was measured every day. The growth curve showed that in EGF + 5-FU group, the tumor grew more slowly than 5-FU group. Error bars correspond to mean ± SD (*: $P < 0.05$).

significantly impacts the malignant progression of colon cancer and thus patient survival and tumor recurrence [28]. Thus, CD133 can be used to identify colorectal CSCs. In our study, we used CD133 as a colorectal CSC marker. Because EGF stimulates cell proliferation [29], we used this molecule to induce cells to enter the cell cycle. We found that CD133+ cells in the EGF-treated group showed higher Ki-67 expression than in the control group, as well as a higher proportion of cells in S + G2/M. The results indicated that EGF stimulated CD133+ CSCs to proliferate, thus inducing the cells to enter the cell cycle. The apoptosis rate of cancer cells increased *in vitro* and the tumor volume of xenografts decreased *in vivo* in the EGF + 5-FU group compared to that in the 5-FU alone group. These results clearly demonstrate that the chemotherapeutic effect was enhanced. In conclusion, EGF can enhance tumor sensitivity to chemotherapeutic drugs, likely by promoting tumor stem cells to enter the cell cycle. However, the specific and detailed mechanisms will be examined in our future studies.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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