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Anti-angiogenesis participates in antitumor effects of metronomic capecitabine on colon cancer

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ABSTRACT

Inhibitory effects and potential mechanisms of capecitabine metronomic chemotherapy on colon cancer were investigated in this study. Metronomic chemotherapy with fluorouracil or capecitabine inhibited proliferation of colon cancer cells both *in vitro* and *in vivo*. Capecitabine metronomic chemotherapy demonstrated equal effects as CTX metronomic chemotherapy. Metronomic capecitabine or CTX chemotherapy decreased vascular endothelial growth factor (VEGF) but elevated thrombospondin-1 (TSP-1) expression, reduced CEP levels and decreased microvessel density (MVD). These results indicated anti-angiogenesis may be correlated with the antitumor effects of metronomic capecitabine in colon cancer.

Introduction

Colorectal cancer is a highly prevalent disease in China. The incidence and mortality of this disease are 29.44 per 100,000 and 14.23 per 100,000, respectively. Its trend of incidence has been increased dramatically in recent years [1]. The evolution and progresses in chemotherapy and molecular targeted therapy have greatly improved overall survival (OS) and progression-free survival (PFS) of metastatic colorectal cancer patients [2]. However, the duration for a new agent from development to marketing is quite long. How to optimize the utility and maximize the efficacy of current available agents are interesting questions.

Traditional chemotherapy strategy for solid tumors is periodically administration with high dose cytotoxic agents to reach clinical therapeutic concentrations (Cmax). For example, Cmax of CTX, 5-Fu and capecitabine in common regimens were 500 mg/m^2 [3], 600 mg/m^2 [4] and 1000 mg/m^2 [5] respectively. Besides the toxicity issues, the residual tumor cells may grow during chemotherapy intervals, resulting in acquired resistance and ultimately leading to treatment failure [6].

OPTIMOX [7], DREAM [8], and CAIRO [9] studies investigated the efficacy and safety of "chemotherapy vacation", "stop-and-go", or "maintenance therapy" modes for the treatment of advanced colorectal cancer. Kerbel et al. [10,11] offered a "metronomic chemotherapy" mode which administrating high-frequency low-dose (one-tenth to one-third of normal dosage) chemotherapeutic agents to inhibit tumor growth. Colleoni et al. [12] reported that high-frequency low-dose cyclophosphamide (CTX) and methotrexate (MTX) in treating metastatic breast cancer achieved good effect and reduced toxicity. Kerbel et al. [13] found that metronomic CTX and vincristine significantly inhibited melanoma xenografts growth. The mechanisms of metronomic chemotherapy are not entirely clear. Kamat et al. [14,15] found that activated endothelial cells were more sensitive to low-dose chemotherapy than tumor cells, which provides the hints that anti-angiogenesis may involved in the metronomic therapeutic model in solid tumors. Related researches have been reported in breast cancer, prostate cancer, and other malignancies [16,17], but rarely in colorectal cancer.

Several large-scale clinical trials have demonstrated non-inferiority of capecitabine, a pro-drug of 5-Fu, to intravenous infusion of 5-Fu in colorectal cancer treatment. Oral administration avoids a tedious peripherally inserted central catheter (PICC) placement and catheter-related complications. Also, oral administration is

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more convenience and easy to maintain certain blood concentration, thus making capecitabine more suitable for metronomic chemotherapy. Kolinsky et al. [18] reported capecitabine regimens in colorectal cancer xenografts were 400 mg/kg/day on day 1–14 of 21 day cycle, or 700 mg/kg/day on day 1–7 of 14 day cycle. These results were based upon the concepts of maximum tolerance and best cytotoxic effects. In this study, capecitabine regimen to treat colon cancer xenografts was 60 mg/kg every 3 days, an acceptable metronomic dose. Both *in vivo* and *in vitro* experiments were performed to evaluate the inhibitory effects of capecitabine metronomic chemotherapy on colon cancer cells, and its potential mechanisms were also investigated.

Materials and methods

Cell lines and culture

Human colon cancer cell lines, HT29, HCT116, SW620, and SW480, and human umbilical vein endothelial cells (HUVEC) were obtained from Shanghai Institute of Digestive Surgery. These cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 and saturated humidity. For subcutaneous xenograft establishment, HCT116 cells were trypsinized and suspended at a concentration of 1×10^7/mL.

Colon cancer xenografts establishment and tissue collection

Male Balb/c nude mice, 4–6 weeks of age, with body weight of 15–20 g, were provided by the Research Center of Experimental Medicine, Shanghai Jiaotong University School of Medicine Affiliated Ruijin Hospital. Prior to performing the experiment, animals were placed in separate cages for 1 week to adapt new environment.

The administration of therapy began when the subcutaneous nodules were about 2 mm in diameter. The mice were randomly divided into following 3 groups: (1) Control group: intra-peritoneal injection of normal saline (NS); (2) CTX metronomic chemotherapy (CTX LDM Group): intra-peritoneal injection of CTX, 20 mg/kg/time [19]; and (3) Capecitabine metronomic chemotherapy (capecitabine LDM group): gavages, 60 mg/kg/time, which was almost one-seventh of reported normal dosage at 400 mg/kg/time [20]. Each group received treatment once every 3 days. The longest diameter (L) and shortest diameter (W) of the tumors were measured with caliper every 7 days, beginning on the first medication day. The tumor volumes (V) were calculated according to the following formula: $V = [(W + L)/(2 \times W \times L)] \times 0.5236$, the tumor growth curves were figured. After 4 weeks of treatment, the mice were sacrificed, and xenografts specimens and peripheral blood samples were collected.

Western blot

Proteins were extracted from the cancer cells by radio-immunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology Companies, USA), and concentrations were determined by bicinchoninic acid (BCA) kit (Pierce, USA). After adding protein loading buffer, the sample (50 μL) was heated at 100 °C for 10 min and separated by 10% Tris–Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins in gels were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 2 h at room temperature. After washing the membrane with PBS, the primary antibodies of thymidine phosphorylase (TP) and thymidine phosphate synthase (TS) were added (1:1000, Abcam Corporation, USA) and incubated overnight at 4 °C. After secondary antibody (1:15000, LI-COR, Nebraska, USA) exploration, the protein bands were scanned by Infrared Imaging Systems.

Immunocytochemistry and immunohistochemistry (IHC) staining

After digestion, the cultured cells were fixed on slides with paraformaldehyde. Immunocytochemistry was performed by TP and TS antibodies with a concentration of 1:500. The TP and TS that stained positive were identified by the appearance of brown particles in nucleus or cytoplasm of tumor cells.

For immunohistochemistry staining, the tissue specimens were fixed in 10% formalin and confirmed to be a tumor by hematoxylin and eosin (HE) staining. Then, EnVision immunohistochemical staining (DAKO Corporation, Denmark) was conducted with following primary antibodies: Ki-67 (1:50, DAKO Corporation, Denmark), CD34 (1:150, Abcam Corporation, USA), VEGF (1:200, Santa Cruz Biotechnology Company, USA), and TSP-1 (1:500, Abcam Corporation, USA). The criteria for the positive staining were as following: appearance of brown particles in nuclei was defined as Ki-67 positive; appearance of brown particles in cytoplasm of the tumor cells was defined as VEGF and TSP-1 positive; appearance of brown particles in endothelial cells was defined as CD34 positive. The MVD quantitation in the highest vascularization was examined in each tumor.

Evaluation of cell proliferation by CCK-8 assay

HCT116 colon cancer cells, growing in logarithmic phase, were seeded into 96-well plates (1×10^3 cells per well). After adherent growth, CTX (0–1600 μg/L) or 5-Fu (0–160 μg/L) were added and incubated for 5 days. The fresh, drug-containing culture medium was replaced every 24 h. Inhibition rates were calculated by measuring the OD450 absorbance values every 24 h with CCK-8 assay (Dojindo Corporation, Japan). The 25% inhibitory concentration (IC25) levels of CTX and 5-Fu were calculated at 48 h and the inhibition rates were calculated at 120 h. The experiments were repeated 3 times.

Evaluation of cell invasion by Transwell assay

HCT116 cells were seeded into small matrigel-covered wells (50000 per well). Two hundred milliliters of CTX (200 μg/L) or 5-Fu (10 μg/L) in serum-free culture medium was added to inner-chamber, and 600 μL of serum-containing culture medium was set to outer-chamber. The number of cells in each well were counted 24 h later under high-powered microscope after crystal violet staining. The experiment was repeated 3 times.

Flowcytometry analysis

Colon cancer cells were seeded in 24-well plates (10^5 cells per well) with the culture medium containing 200 μL of CTX (200 μg/L) or 5-Fu (10 μg/L). Twenty-four hours later, the cells were collected and stained by Annexin V-FITC (BD Biosciences Corporation, USA). Cell cycle and apoptosis were analyzed by Fluorescence Assisted Cell Sorting (FACS).

For the detection of circulating endothelial progenitor cells (CEPs) in the peripheral blood, the blood samples of animals were pre-treated with EDTA and washed thoroughly to remove dead cells, platelets, and cell debris. CEP subgroups (at least 5×10^4 cells) were marked by following monoclonal antibodies: CD117, Flk-1 (VEGFR-2), and Sca-1 (eBioscience, USA), and detected by FCS500 MC to determine the percentage of stained cells. It was considered an effective test if the CEP counting window collected at least 50 cells.

HUVEC tubule formation assay

HUVEC cells were seeded in matrigel-coated 96-well plates (5000 per well). Two hundred milliliters of serum-free culture medium containing CTX (200 μg/L) or 5-Fu (10 μg/L) was added into the wells. The cultures were incubated at 37 °C with 5% CO_2 for 8 h, and washed with PBS to remove dead and non-adherent cells. The tubule number, total length, and connection points between the tubules were counted under high-powered microscope. The entire experiment was repeated 3 times.

Angiogenic factors detection by enzyme-linked immunosorbent assay

HCT116 cells were seeded in 24-well plates (10000 per well). After completely adherenence, the culture medium containing CTX (200 μg/L) or 5-Fu (10 μg/L) was replaced. The cultures were incubated at 37 °C with 5% CO_2 for 8 h. VEGF and TSP-1 levels in supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA). VEGF and TSP-1 in peripheral blood of animals was also determined by ELISA kit.

Statistical analysis

Data are expressed as mean and standard deviation ($\bar{x} \pm S$), and all the statistics analyses were performed by using SPSS17.0 (SPSS Inc.; Chicago, IL, USA). The tumor growth curves were analyzed by Mann–Whitney U test. P value less than 0.05 was considered as statistically significant.

Results

Metronomic treatment inhibited proliferation and invasion of colon cancer cells in vitro

TP and TS protein expression in four colon cancer cell lines were detected by western blot and immunocytochemistry staining. HCT116, a colon cancer cell line with high TP and low TS expression, was selected for further experiments (Fig. 1).

CCK-8 assay was used to identify the inhibitory effects of CTX and 5-Fu on the proliferation of HCT116 cells. IC25 of CTX and 5-Fu were 162.12 ± 15.03 μg/L and 8.53±0.86 μg/L respectively (Table 1), so 200 μg/L of CTX and 10 μg/L of 5-Fu were used as the metronomic dosage.