Promotion of tumor progression induced by continuous low-dose administration of antineoplastic agent gemcitabine or gemcitabine combined with cisplatin

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Research

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Abstract

Background: There are indications that certain antineoplastic agents at low dosages may exhibit abnormal pharmacological actions, such as promoting tumor growth. However, the phenomenon still needs to be further confirmed, and its underlying mechanisms have not yet been fully elucidated.

Methods: Gemcitabine (GEM) and cisplatin (CDDP) were employed as representative antineoplastic agents to observe effects of continuous low-dose chemotherapy with GEM or GEM combined with CDDP (GEM+CDDP) on tumor formation and growth

in xenograft tumor models *in vivo*. Tumor and endothelial cell functions, apoptosis, cell cycle analysis, as well as bone marrow derived cells (BMDCs) mobilization, were evaluated with transwell, MTT or flow cytometry analysis *in vitro*, respectively. Histological methods were employed to assess angiogenesis in tumor tissues.

Results: The results showed that tumor formation and growth were both significantly promoted by GEM or GEM+CDDP at as low as half of the metronomic dosages, which were accompanied by enhancements of angiogenesis in tumor tissues and the release of proangiogenic BMDCs in the circulating blood. Additionally, GEM or GEM+CDDP at low concentrations dramatically facilitated the proliferation, migration, and invasion of tumor cells *in vitro*. Cell-cycle arrest, activation of associated apoptotic proteins, and inhibition of apoptosis were also observed in tumor cells.

Conclusions: These findings indicate that, the continuous low-dose administration of GEM and GEM+CDDP can promote tumorigenesis and tumor progression *in vivo* by inhibiting apoptosis, mobilizing BMDCs, and promoting angiogenesis in certain dose ranges. These findings urge further investigations to avoid the potential risks in current empiric continuous low-dose chemotherapy regimens with antineoplastic agents.

Major Finding

This study observes a previously neglected pharmacological phenomenon and investigates its mechanism of that the continuous low-dose administration of some antineoplastic agents in certain dose ranges can promote tumorigenesis and tumor progression *in vitro* and *in vivo*, through stimulation of tumor cell functions directly as well as enhancement of tumor angiogenesis by BMDCs recruitment indirectly. The results alert to a potential risk in current empirically based continuous low-dose chemotherapy regimens such as metronomic chemotherapy.

Background

Chemotherapy still plays a pivotal role in current cancer treatments (1). However, the conventional chemotherapy with antineoplastic agents at the maximum tolerated dose (MTD) is typically administrated in short cycles, obligatorily separated by long treatment breaks (e.g., 3 weeks), and

unfortunately accompanied by serious toxic side effects. The prominent toxicities, or even death, associated with high doses used in these schedules often restrict the ability to increase chemotherapeutic dosages and also impair treatment outcomes (2, 3). This situation has led to the introduction and clinical testing of some high-frequency low-dosage regimens, such as metronomic chemotherapy and adaptive therapy to reduce toxicity and obtain optimum outcomes (4). Metronomic chemotherapy, characterized by continuous and dose-dense administration of chemotherapeutic drugs with lowered doses, is usually associated with better tolerance than conventional chemotherapy, and favorable response rates have been reported in various settings (5). Therefore, pre-clinical and clinical studies have been investigating the use of such low dose metronomic therapy as an augmentation or as a substitute for conventional regimens (6). However, numbers of fundamental issues in this field relating to pharmacology or clinical applications have not yet been clarified. As a result, the clinical development of metronomic chemotherapy has been impeded by numerous limitations, including the lack of its basic pharmacological data, ambiguity of its definition and the resulting empiric design of treatment protocols. The current metronomic chemotherapy protocols applied clinically are exclusively empirical in terms of dosing (e.g., half, one third, one tenth of the maximum tolerated dose) and scheduling (e.g., weekly, twice a week, thrice a week, daily) due to the lack of theory-based dose setting method.

Cancerous cells can lie dormant for years before initiating tumor outgrowth again (7, 8). It is generally believed that there are many dormant microtumors in normal tissues. Meanwhile, tumor recurrence and metastasis after chemotherapy represents the main cause of cancer-related mortality (9). Though there is a lifelong risk of recurrence and metastasis, the risk of recurrence typically peaks during the first 5 years after treatment, and especially during the first 2 years (10). Cancer can recur in the same place as the original tumor or in other places in the body if the tumor cells spread. Moreover, it has been found that the recurrent tumor tissues may not be homologous with the primary tissue, suggesting that recurring tumors are more likely to derive from independent sources such as dormant microtumors (11). However, little is known about what triggers steady state original tumor cells to reawaken, as well as the causative factors or causes of these dormant non-homologous microtumor progression.

Antineoplastic agents at certain low dosages have been found to enhance tumor development in some animal models (12–17). For example, low-dose bleomycin was reported to promote tumor metastasis and low-dose cyclophosphamide was found to enhance both tumor growth and metastasis in mice (12, 18). It was also found that antineoplastic agents are carcinogenic even at very low doses (19, 20). Healthcare workers exposed to antineoplastic agents polluted the environment suffer significantly increased fetal malformations, chromosomal abnormalities, and tumor occurrence (21, 22). Moreover, studies have revealed that low-concentration antineoplastic agents can enhance the proliferation, adhesion, and migration of endothelial cells, suggesting that these agents promoting tumor angiogenesis and tumor progression (23).

Therefore, the question that naturally arises is whether a tumor's relapse is *via* activation of dormant microtumors by chemotherapy. Therefore, it is necessary to determine whether inappropriate

chemotherapy regimens can lead to adverse treatment outcomes. For instance, the potential role of low-dose antineoplastic agents in tumor recurrence and metastasis.

GEM is powerful antineoplastic drug that is used for pancreatic cancer and breast cancer chemotherapy, either alone or in combination with several types of cytotoxic drugs [e.g., CDDP and paclitaxel], and is also an option in various other solid and hematological cancers (24, 25). Importantly, GEM is one of the most commonly used antineoplastic agents in metronomic chemotherapy for solid cancers such as breast, liver and pancreatic cancer (26). However, very few studies have investigated the dose effect relationship of GEM and its combined chemotherapy under continuous low-dose administration conditions such as metronomic chemotherapy. Therefore, in the present study, GEM and GEM plus CDDP were selected as the observation objects to investigate the effects of continuous low-dose administration of antineoplastic agents on tumorigenesis and tumor progression *in vivo*. The pharmacological effects of low-concentration GEM or GEM + CDDP on the functions and apoptosis of tumor and endothelial cells were also investigated *in vitro*.

Material And Methods

Chemicals and antibodies

Pharmaceutic grad GEM (Cat.H20030104) and CDDP (Cat.H20073652) were purchased from Hanson Pharma and Qilu Pharmaceutical Co., Ltd (China), respectively. All-trans retinoic acid (ATRA) (Cat.H10970053) was obtained from Shandong Liangfu Pharmaceutical Co., Ltd (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Cat.M2128) and dimethyl sulfoxide (DMSO) (Cat.D2650) were from Sigma (USA). Annexin V-PI Detection Kit was obtained from BD Bioscience (USA). PI staining Kit was purchased from Beyotime Biotechnonology (China). Primary antibodies for β -actin (Cat.20536-1-AP), Bcl-2 (Cat.60178-1-Ig), Bax (Cat.50599-2-Ig), Caspase-3 (Cat.19677-1-AP), Caspase-9 (Cat.10380-1-AP) were obtained from Proteintech (USA). Cleaved-caspase-3 (Cat.YC0013) and Cleaved-Caspase-9 (Cat.YC0006) were purchased from Immunoway (China). PE-anti-mouse Gr-1 (Cat.108407), FITC anti-mouse CD11b (Cat.101205), and FITC anti-mouse CD61 (Cat.104305) were obtained from Biolegend (USA). The laminin antibody (Cat.ab11575), GFP antibody (Cat.ab6556) and CD31 antibody (Cat.ab18298) were ordered from Abcam (UK).

Animals and cells lines

8-week-old C57BL/6J mice were acquired from Animal Experiment Center, Xi'an Jiaotong University. 8-week-old BALB/C nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. All animals were housed under constant temperature, humidity, and lighting (12-h light per day) and allowed free access to food and water. All animal experiments were approved by the ethical committee for animal care of Xi'an Jiaotong University.

MCF-7, T-47D human breast cancer cell lines, and endothelial cells were purchased from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The B16 cell line was

a gift from the Laboratory of Urology, Xi'an Jiaotong University. Cells were cultivated in recommended medium, supplemented with 10% FBS, 1% penicillin, and streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Xenograft tumor model *in vivo*

42 female BALB/C nude mice were used to establish *in vivo* subcutaneous tumor model. In brief, 1×10^5 MCF-7 cells were injected subcutaneously into the flank of each mouse. Mice were then randomized into seven groups (n = 6/group): control, 1 mg/kg GEM, 4 mg/kg GEM, 0.05 mg/kg CDDP, 0.2 mg/kg CDDP, 1/0.05 mg/kg GEM + CDDP and 4/0.2 mg/kg GEM + CDDP. For C57BL/6J mice, 1×10^6 B16 cells were injected subcutaneously into the flank of each mouse, and then randomly assigned to control or treatment groups (n = 8/group).

To mimic chemotherapy, GEM or GEM + CDDP was administered 24h after tumor xenograft and repeated every 48h. 0.9% sodium chloride solution was delivered parallelly as control. Each administration was divided into two 0.2mL injections with drug or saline *via* the abdominal route. Tumor volumes were determined every 2 days in C57BL/6J mice and every 5 days in BALB/C nude mice with the volumetric calculation formula: $\text{length} \times \text{width}^2 \times 0.5$. At each time point, mice were anesthetized with 20% urethane, and blood was collected from the vena cava with heparin anticoagulation. Tumors were harvested by detaching the surrounding connective tissue 2 weeks in C57BL/6J mice or 6 weeks in BALB/C nude mice post-implantation, respectively by detaching the surrounding connective tissue, weighed, and processed for immunohistochemistry.

Collection and isolation of bone marrow cells

Mice were anesthetized with urethane and then euthanized immediately. Bone marrow was extracted from the femurs and tibias of mice. A single bone marrow cell suspension was prepared by passing the bone marrow through a 21-gauge needle. Then the cells were pelleted by centrifugation and suspended in medium for bone marrow transplantation in further experiments at a density of 1×10^7 cells/mL.

Bone marrow transplantation

Recipient mice (8 weeks old) were lethally irradiated (9Gy) followed by bone marrow reconstitution by tail vein injection with 1×10^7 bone marrow cells isolated from green fluorescent protein-positive (GFP⁺) donor femurs. 8 weeks after bone marrow transplantation, the mice were used for tumor experiments.

MTT assay

Cells (3×10^4 /mL) were seeded in 96-well plates. 20μL of MTT (5mg/mL) was added into each well and incubated for 4h. Then, 150μL of DMSO was added into each well to solubilize formazan for 15min. The absorbance was determined at 490nm on a microplate reader (BioTek). Each experiment was repeated three times.

Clone assay

1×10^2 cells were seeded in triplicate wells of 6-well plate ($n = 3$) and cultured for 2 weeks. After fixed with 4% paraformaldehyde for 20min, the colonies were stained using 0.1% crystal violet and counted.

Transwell migration and invasion assay

Cell migration and invasion were assessed using 8- μ m-pore transwell compartments (Corning). 5×10^5 cells were suspended in serum-free medium in the upper compartment. The translocated cells were stained with 0.5% crystal violet for 20 min at 25°C after cells were incubated at 37°C for 24h. For invasion assays, Matrigel Matrix (BD Biosciences) was added to each well according to the manufacturer's instructions before 5×10^5 cells were suspended in the upper compartment. After cells were incubated at 37°C for 24h, the translocated cells were incubated with 0.5% crystal violet for 20min at 25°C, and were counted under an upright microscope (five fields each chamber). Each assay was repeated in three independent experiments.

Wound-healing assay

Cells were plated in triplicate into 6-well plates. A standard 10 μ L pipette tip was used to scratch wound when the cells reached a density of 95%. Subsequently, the cells were cultured in FBS-free medium or treated with drugs. After 24h, the wound closure was captured by a microscope and calculated using the software of Image J (National Institutes of Health, Bethesda, MD, USA).

Cell cycle analysis

Cells were harvested 24h after treatments and fixed in ice-cold 75% ethanol overnight at 4°C. Cells were treated with PI staining Kit according to the manufacturer's instructions after washing with PBS for two times, and then analyzed by FACS-Canto II flow cytometry (Becton Dickinson Company).

Apoptosis analysis

Cells were harvested 48h after treatments, washed and resuspended with cold PBS. The staining process was then conducted using the Annexin V-FITC/PI Apoptosis Detection Kit. The stained cells were analyzed using FACS-Canto II flow cytometry (Becton Dickinson Company).

Western blotting analysis

Cells were lysed with RIPA buffer containing protease inhibitor on ice. Then equal amounts of protein lysates were electrophoretically separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). After blocking with 5% nonfat dried milk for two hours, the membranes were incubated with primary antibodies overnight at 4°C. Another incubation with a horseradish peroxidase-conjugated secondary antibody was performed in the following day for two hours at 37°C, after when the

protein bands were detected using the Super Signal West Pico kit (Thermo Fisher Scientific). All Western blot experiments were repeated at least three times.

Histology and immunohistochemistry

Tissues were embedded in OCT and snap frozen in liquid nitrogen or fixed in 10% buffered formalin followed by paraffin embedding. IHC staining was applied to 3- μ m thick sections of paraffin-embedded tissue specimens with a PV-9001 Detection Kit (ZSGB-BIO). Briefly, formalin-fixed paraffin-embedded tissues were deparaffinized in xylene for 10min and rehydrated in a graded series of ethanol solutions. The tissues were immersed in 0.01M citric acid buffer at 121°C for 5min and then cooled and washed with 0.1M PBS for 3 times. After treated with 3% hydrogen peroxide for 10min, the tissues were incubated with primary antibodies overnight at 4°C, followed by the secondary antibody for 30min at 25°C. Sections were stained with 3,3'-diaminobenzidine and counterstained using hematoxylin for 5 seconds, dehydrated in a graded series of ethanol solutions, immersed in xylene, and examined with a microscope (Axio Image M2, Zeiss). The microvascular area (MVA) in IHC stained images of each group was analyzed with Image-Pro Plus software.

Flow cytometry

Cells isolated from circulating blood were incubated with fluorescein isothiocyanate (FITC) anti-mouse CD11b antibody, PE anti-mouse Gr-1, and PE anti-mouse CD61, following a standard laboratory protocol (27). The cells were subjected to flow cytometer on FACS-Canto II flow cytometry (Becton Dickinson Company), and data were analyzed with Cell Quest Software.

Statistical analysis

Statistical analysis was performed using SPSS software. Data were expressed as mean $\pm$ standard deviation values. One-way ANOVA or Fisher's exact test was used for comparison tests. Probability values of $P < 0.05$ were considered statistically significant.

Results

Continuous low-dose administration of GEM and/or CDDP facilitated tumor formation and promoted tumor growth *in vivo*

Surprisingly, we found that continuous low-dose administration of GEM and GEM + CDDP significantly promoted tumor growth in xenograft tumor models *in vivo*.

As shown in Fig. 1a-c, in a B16 tumor model, once every 48h administration of 1.25 mg/kg GEM and 0.6/0.03 mg/kg GEM + CDDP for 2 weeks remarkably induced in B16 tumor growth by 45.2% and 32.2%, respectively, relative to control (each $p < 0.05$). In contrast, once every 48h administration of 5 mg/kg GEM, 2.4/0.12 and 4.8/0.24 mg/kg GEM + CDDP for 2 weeks markedly inhibited tumor growth. Of note, no significant effect was found in tumor treated with CDDP alone. In addition, the average tumor weights

in the 5 mg/kg GEM and 4.8/0.24 mg/kg GEM + CDDP groups after 2 weeks treatment were reduced by 36.6% and 39.1%, respectively, relative to control (both $p < 0.05$). Strikingly, in MCF-7 mediated nude mouse tumor model, out of six tested mice, we found some tumors (from 1 to 4) developed from either GEM or GEM + CDDP low-dose treatment groups but not in the control group 6 weeks after implanting equal number of MCF-7 tumor cells (Fig. 1d). Together these findings indicate that the continuous low-dose administration of GEM and GEM + CDDP can promote significant tumor formation and growth *in vivo*.

Low-concentration GEM and GEM + CDDP promoted B16, MCF-7, and T-47D cell functions *in vitro*

Based on the surprising *in vivo* findings, we intended to test the effects of GEM and CDDP on the proliferation of B16 cells, MCF-7 and T-47D tumor cells. The cell proliferation was measured by MTT assay. As shown in Fig. 2a, low-concentration GEM (1×10^{-3} or 5×10^{-5} μM) and GEM + CDDP treatment promoted the proliferation of MCF-7 cells, whereas 10 or 0.5 μM GEM and GEM + CDDP significantly suppressed cell proliferations (both $p < 0.01$). In contrast, no significant effect was found in CDDP-treated B16 or MCF-7 cell. These results implied that levels of antineoplastic agents used have a significant impact on the fate of tumor cells, e.g., proliferation or cell death.

Meanwhile, although the low concentrations of 1×10^{-5} μM GEM or 5×10^{-7} μM CDDP did not affect the proliferation of T-47D tumor cells alone, their combination at these concentrations resulted in marked facilitation ($p < 0.05$) (Fig. 2b). Similar phenomena were found in B16 cells with 1×10^{-3} μM GEM and 5×10^{-5} μM CDDP (note that these concentrations differ from those applied to T-47D cells) ($p < 0.05$) (Fig. 2c).

We also performed the colony formation and wound healing assays. As shown in Fig. 2d, colony formation was markedly enhanced in B16 cells at 1×10^{-3} μM GEM and $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP groups relative to control (both $p < 0.01$). Similar results were seen in the wound-healing assay showing that low-concentration 1×10^{-3} μM GEM or $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP promoted the proliferation of B16 cells (both $p < 0.01$) (Fig. 2e).

The effects of GEM or GEM + CDDP on the invasion and migration functions of B16, MCF-7 and T-47D cells were investigated at low concentrations. The results are shown in Fig. 2f-h, relative to control, the invasion and migration of tumor cells were significantly enhanced in MCF-7 cells after 1×10^{-3} μM GEM and $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP treatment, in T-47D cells after 1×10^{-5} μM GEM and $1 \times 10^{-5}/5 \times 10^{-7}$ μM GEM + CDDP treatment, and in B16 cells after 1×10^{-3} μM GEM and $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP treatment (each $p < 0.01$). These results indicate that both GEM and GEM + CDDP can enhance the tumor-cell functions at low-concentration *in vitro*.

In addition, the cell-cycle assay revealed that 1×10^{-3} μM GEM and/or 5×10^{-5} μM CDDP markedly decreased the fraction of MCF-7 and B16 cells at the G2 and M phases of the cell cycle, while 1×10^{-5} μM

GEM and/or 5×10^{-7} μM CDDP decreased the fraction of T-47D cells at the G0 and G1 phases (each $p < 0.01$) (Fig. 2i-k).

Low-concentration GEM and GEM + CDDP inhibited apoptosis of B16, MCF-7, and T-47D cells *in vitro*

In a standard culture condition, we observed approximately 10–25% apoptotic cells in B16, MCF-7, and T-47D cells. Intriguingly, when we treated tumor cells with low-concentration of GEM and GEM + CDDP (1×10^{-5} μM and $1 \times 10^{-5}/5 \times 10^{-7}$ μM , respectively), the apoptotic cells were profoundly declined by 44.1% and 68.9%, respectively, relative to non-treated MCF-7 cells (both $p < 0.01$), by 41.1% and 55.4% in B16 cells ($p < 0.01$ and $p < 0.05$ respectively), and by 50.4% and 67.4% in T-47D cells (both $p < 0.01$) (Fig. 3a-c). In addition, MCF-7 cells after 1×10^{-3} μM GEM and $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP treatment increased the antiapoptotic protein Bcl-2 by 92.1% and 136.2%, while decreased the proapoptotic Bax protein by 35.2% and 53.3%, respectively, compared to non-treated cells. Furthermore, caspase-3 expression levels in MCF-7 cells were markedly decreased by 29.8% and 68.6%, cleaved-caspase-3 were decreased by 34.9% and 54.5% after treatment with 1×10^{-3} μM GEM and $1 \times 10^{-3}/5 \times 10^{-5}$ μM GEM + CDDP, respectively, compared to non-treated cells. Also, caspase-9 expression levels were decreased by 48.1% and 76.2%, and cleaved-caspase-9 were decreased by 15.6% and 43.9%. Similarly results were seen in low-dose GEM and GEM + CDDP treated T-47D cells, 1×10^{-5} μM GEM and $1 \times 10^{-5}/5 \times 10^{-7}$ μM GEM + CDDP treatment increased Bcl-2 expression by 7.3% and 172.7%, respectively, and decreased Bax expression by 55.5% and 55.6%, caspase-3 expression by 17.2% and 20.3%, cleaved-caspase-3 expression by 41.2% and 63.4%, caspase-9 expression by 31.1% and 27.8% and cleaved-caspase-9 expression by 16.9% and 36.8%, respectively, compared to non-treated cells (Fig. 3d-e).

Those findings strongly suggest that the tumor growth acceleration induced by low-concentration GEM and GEM + CDDP is associated with inhibition of the expression of proapoptotic proteins and promotion of the expression of antiapoptotic proteins.

Low-dose GEM and GEM + CDDP promoted tumor angiogenesis *in vivo*, and inhibited the proliferation of endothelial cells *in vitro*

As shown in Fig. 4a-b, the areas of CD31⁺ vessels in 1.25 mg/kg GEM and 0.6/0.03 mg/kg GEM + CDDP groups were significantly enhanced in B16 tumor tissues relative to control (both $p < 0.01$), as was also the case in laminin positive vessels (both $p < 0.01$). This implies that GEM and GEM + CDDP can promote tumor angiogenesis under continuous low-dose administration conditions. However, we found that GEM and GEM + CDDP inhibited endothelial cells proliferation in the concentration range from $1 \times 10^{-2}/5 \times 10^{-4}$ μM to 10/0.5 μM *in vitro* (each $p < 0.01$), with no stimulation role of GEM or GEM + CDDP found at lower concentrations (Fig. 4c).

These results suggest that GEM and GEM + CDDP can promote angiogenesis in B16 tumor tissues at low-dosage *via* pathways independent of endothelial cells stimulation.

Continuous low-dose administration of GEM and GEM + CDDP promoted the mobilization of Gr-1⁺CD11b⁺ and CD61⁺BMDCs

To further explore the potential mechanisms of low-dosage GEM and GEM + CDDP induced angiogenesis, we measured the proangiogenic BMDCs, e.g., Gr-1⁺CD11b⁺ and CD61⁺BMDCs. As shown in Fig. 5a, the proportions of Gr-1⁺CD11b⁺ BMDCs were increased by 52.3%, 53.1% and 17.7% in 1 mg/kg GEM, 4 mg/kg GEM and 0.2 mg/kg CDDP groups, respectively, compare to saline control group. The proportions of Gr-1⁺CD11b⁺ BMDCs were increased by 331.7% and 406.2% in the 1/0.05 and 4/0.2 mg/kg GEM + CDDP groups respectively, relative to control (both $p < 0.01$). Combination of GEM and CDDP significantly enhanced the mobilization of Gr-1⁺CD11b⁺BMDCs compared to GEM or CDDP alone (all $p < 0.01$). The Gr-1⁺CD11b⁺ and CD61⁺BMDCs counts were increased by 65.1% and 18.6%, respectively, in B16 tumor bearing C57BL/6J mice after 2 weeks treatment with 1.25 mg/kg GEM (both $p < 0.05$) (Fig. 5b-c). The average proportions of Gr-1⁺CD11b⁺ and CD61⁺BMDCs in the 5 mg/kg GEM group also increased slightly, but the change did not reach statistical significance relative to control group. The Gr-1⁺CD11b⁺BMDCs count in 0.6/0.03 mg/kg GEM + CDDP-treated B16-tumor-bearing C57BL/6J mice was markedly increased by 23.5% relative to control ($p < 0.05$) (Fig. 5d). The numbers of CD61⁺BMDCs increased by 5.3% and 10.9% in the 0.6/0.03 and 4.8/0.24 mg/kg GEM + CDDP groups, respectively, relative to control group (both $p < 0.05$) (Fig. 5e).

These results indicate that continuous low-dose administration of GEM and GEM + CDDP can significantly mobilize proangiogenic Gr-1⁺CD11b⁺ and CD61⁺BMDCs into the circulating blood in tumor-bearing mice models.

Continuous low-dose administration of GEM and GEM + CDDP promoted the recruitment of BMDCs in tumor tissues

Tumor growth in C57BL/6J mice with GFP bone marrow was observed after 4-weeks of treatment with frequency of once every 2 days with antineoplastic agents. Tumor weights in the 1.25 mg/kg GEM and 0.6/0.03 mg/kg GEM + CDDP groups were increased by 342.5% and 344.9%, respectively, relative to control (both $p < 0.01$), while they were decreased by 61.8% and 12.9% in the 5 mg/kg GEM and 4.8/0.24 mg/kg GEM + CDDP groups, respectively (Fig. 6a).

The densities of GFP⁺BMDCs were analyzed using immunohistochemically of tumor tissue sections. Compared with the control group, the numbers of GFP⁺ cells were increased by 185.8% and 227.8% after treatment with 1.25 mg/kg GEM and 0.6/0.03 mg/kg GEM + CDDP, respectively, compared to control group (both $p < 0.01$) (Fig. 6b).

These results indicate that the continuous low-dose administration of GEM and GEM + CDDP can promote the recruitment of GFP⁺ BMDCs in tumor tissues.

Continuous low-dose administration of GEM and GEM + CDDP promoted the expression of proangiogenic proteins in tumor tissues

To confirm the role of low-dose GEM and GEM + CDDP in promoting angiogenesis, we also surveyed the expression changes of some angiogenic proteins in B16 tumor tissues by Immunoblotting. As shown in Fig. 7, the protein expression levels of MMP-9, VE-cadherin, VEGFR₁, and VEGFR₂ in the 1.25 mg/kg GEM group were increased by 66.6%, 124.3%, 87.6%, and 54.5%, respectively, relative to control (each $p < 0.01$). In addition, except MMP-9, the protein expression levels of VE-cadherin, VEGFR₁, and VEGFR₂ in tumor tissues were significantly increased by 65.0%, 30.0%, and 25.0%, respectively, after treatment with 0.6/0.03 mg/kg GEM + CDDP as compared with control (each $p < 0.05$).

These results suggest that the continuous low-dose administration of GEM and GEM + CDDP can promote the expression of proangiogenic proteins.

ATRA inhibited enhancement of B16 tumor growth and mobilization of BMDCs induced by continuous low-dose administration of GEM

As shown in Fig. 8a, while 1.25 mg/kg GEM treatment resulted in a 75.7% increase in tumor weights relative to control ($p < 0.01$), co-treatment with 30 mg/kg ATRA led to a 71.2% decrease relative to GEM treatment alone ($p < 0.01$). Similarly, compared to increased Gr-1⁺CD11b⁺ and CD61⁺BMDCs counts in 1.25 mg/kg GEM group, co-treatment with 30 mg/kg ATRA profoundly inhibited Gr-1⁺CD11b⁺ counts 35.8%, and CD61⁺BMDCs counts by 79.1% ($p < 0.01$) (Fig. 8b). Meanwhile, as shown in Fig. 8c, the areas of laminin positive vessels in 1.25 mg/kg GEM were significantly enhanced and decreased in 30 mg/kg ATRA in B16 tumor tissues relative to control (both $p < 0.01$).

These results suggest that low-dose GEM induces mobilization of proangiogenic BMDCs.

Discussion

Low-dose chemotherapy such as metronomic chemotherapy with main advantages of no prolonged drug-free breaks, the potential for delayed development of resistance, low toxicity profile, and convenient use makes it a desirable alternative in clinical applications to overcome the shortcomings of efficacy and toxicity in conventional chemotherapy (28, 29). Nevertheless, owing to the investigational nature of this approach, its regimens are highly empirical in terms of the optimal dose and schedule for the drugs administered. Therefore, greater knowledge of the dose-effect relationship of metronomic chemotherapy is critical to the success of this treatment strategy. However, few studies have focused on the dose-response relationships of low-dose antineoplastic agents, even though clues had shown anomalous pharmacological actions in certain antineoplastic agents under such conditions (30). In present study, both *in vitro* and *in vivo* data suggest that inappropriate antineoplastic agents' dosage and treatment regimen setting may lead to opposite therapeutic outcomes. Therefore, inappropriate low-dose chemotherapy regimens may lead to the risk of adverse outcome, which is often ignored, especially under

the condition of empirical setting of treatment. It should be noted that the results of present study do not negate the low-dose chemotherapy strategies, but call for more dose-response relationship studies and mechanisms understanding of various antineoplastic agents.

Few studies by our groups and others (16) have implied that antineoplastic agents might promote or induce tumor growth under certain low-dose conditions, these phenomena were thus investigated *in vivo* in the present study with lower dose GEM and/or CDDP. GEM is applied to solid tumors with common murine *in vivo* single dose of 100-120mg/kg and clinical dose of 25 mg/kg (1000 mg/m²) (31, 32). Numerous treatment schedules of metronomic GEM have been applied in mice, from continuously 1 mg/kg/day for 28 days to continuous 3.3 mg/kg/day for 21 days (31, 33–35). In order to investigate the aberrant dose-effect relationship of low-dose GEM and tumor growth, as well as considering the current status of empirical dose setting for metronomic chemotherapy in clinic, effects of GEM on tumor growth were observed in the present study at dose ranges below or above the reported dose of metronomic chemotherapy (36, 37). As a result, the single GEM dosage in continuous low-dose administration *in vivo* were set as about half to twice these doses in the mice model in the present study. The concentrations of promoting tumor cell function in the culture medium were selected as the GEM experiment concentrations *in vitro* (Fig. 2a). The dose ratio of GEM to CDDP was set according to the reported clinical dosages (38). The results verified that GEM could exert inhibition effects at high-doses (equivalent to one to two times of the metronomic doses every 2 days) or promotion effects at low-doses (equivalent to half of the metronomic doses every 2 days) on tumor growth in tumor-bearing mice model. A similar biphasic effect dose-response relationship was observed in GEM + CDDP groups *in vivo*.

The present study also found that the antineoplastic agents GEM either alone or in combination with CDDP stimulated the functions and inhibited the apoptosis of tumor cells at low concentrations, indicating a direct role in promoting tumor growth. Previous studies have reported that CDDP enhanced the adhesion of endothelial cells and thereby might promote tumor angiogenesis (23). Nevertheless, no significant promotion effects of CDDP alone on either tumor growth *in vivo* or tumor cell functions *in vitro*, as well as endothelial cells proliferation, were observed at the selected low dosages or concentrations in the present study. However, when CDDP was applied in combination with GEM at low dosages *in vivo* or low concentrations *in vitro*, it demonstrated an enhancement effects on tumor cell proliferation and survival than GEM alone. These results suggest that combination of antineoplastic agents may shift the drugs' optimal concentration and a completely adverse outcome. Therefore, the present experimental results strongly support that dosage setting in chemotherapy regimen should be based on precise sources and bases, so as to ensure dosages and schedules within the therapeutic window. Thus, it could avoid inconsistent or opposite therapeutic effects of empiric metronomic chemotherapy with the treatment goals.

It is widely recognized that dormant microtumors are present in normal tissues, which may be activated and result in cancer progression under certain stimulation conditions (7, 8). Moreover, studies have often found that genotypes of recurring tumors might differ from that of the primary tumor, implying that a heterogeneous tumor probably originated from sources other than primary tumor (11). When nude mice

were subcutaneously inoculated with 1×10^5 MCF-7 tumor cells, the number of cells required was slightly smaller than normal. Then stimulation with low-dose GEM and/or CDDP for 6 weeks distinctly increased the numbers of tumor formation from zero (out of six) in the control group to two in the GEM group and four in the GEM + CDDP group. These results indicate that low-dose antineoplastic agents might promote the formation and progression of residual primary tumor or dormant microtumor. Given the fact that occupational exposure to low-dose antineoplastic agents increases the incidence of tumors, these results consequently support the speculation that that improper low-dose chemotherapy regimens lead to the activation of dormant microtumors by antineoplastic agents, which may promote tumor recurrence and metastasis. Similar phenomena have been reported in tumor radiotherapy, in which low-dose radiotherapy promoted tumor recurrence (39). The present data suggest that there is an urgent need to further investigate the pharmacological responses of antineoplastic agents in clinical applications, as well as the role of antineoplastic agents in tumor relapse, in continuous low-dose regimens such as metronomic chemotherapy.

Angiogenesis plays a key role in the development of solid tumors (40). It has been demonstrated that some antineoplastic agents can directly promote the proliferation, adhesion, and migration of endothelial cells at certain concentrations *in vitro*, thereby promoting tumor angiogenesis (23). The present study found that antineoplastic agents GEM and CDDP either alone or in combination inhibited endothelial cell functions *in vitro* and stimulated angiogenesis *in vivo*. Tumor angiogenesis is a complex multi-step process regulated by various factors and orchestrated by a number of intersecting pathways (41). Thus, even though the endothelial-cell functions were suppressed by antineoplastic agents directly in the present study, the ultimate outcome of tumor angiogenesis still might be enhanced through an indirect pathway. We previously found that BMDCs recruited into angiogenic local tissues played a dominant role in promoting angiogenesis (42, 43). There is also evidence that antineoplastic agents stimulate the mobilization of proangiogenic BMDCs and their release from bone marrow, and enhance the recruitment of BMDCs in tumor tissues (14, 44). The retention of proangiogenic BMDCs in local tissue can then promote tumor angiogenesis *via* the release of stimulating factors (43). The cytometer flow analysis results obtained in this study showed that the antineoplastic agents GEM and GEM + CDDP at low doses significantly increased the circulating level of proangiogenic Gr-1⁺CD11b⁺ and CD61⁺BMDCs. The densities of GFP⁺ BMDCs in tumor tissue sections obtained from mice with bone-marrow transplantation were higher for treatments with GEM and GEM + CDDP than in the control group, indicating that low-dose GEM and GEM + CDDP can promote the recruitment and retention of BMDCs in tumor microenvironment tissues. The expression of proangiogenic receptors such as VEGFR₁ and VEGFR₂ and proangiogenic proteins such as MMP-9 and VE-cadherin in tumor tissues also were found to be promoted by low-dose GEM and GEM + CDDP, which suggested an enhancement role of these antineoplastic agents in angiogenesis, too. Thus, the promotion of tumor angiogenesis by low-dose GEM or GEM + CDDP might be associated with the recruitment of proangiogenic Gr-1⁺CD11b⁺ and CD61⁺BMDCs and changes in the tumor microenvironment indirectly. It is speculated that occupational injuries of cancer incidence increase caused by antineoplastic agents may share similar mechanisms.

Previous studies have shown that ATRA combined with fluorouracil can significantly inhibit tumor growth *in vivo* (45). It was also reported that ATRA induced immature myeloid cells to differentiate into mature dendritic cells, macrophages, and granulocytes, as well as greatly reduced the number of Gr-1⁺CD11b⁺BMDCs in tumor tissues (46, 47). The present study found that 30 mg/kg ATRA markedly inhibited the enhancement tumor growth induced by 1.25 mg/kg GEM and decreased the release of Gr-1⁺CD11b⁺ and CD61⁺BMDCs in the circulating blood. These findings suggest that ATRA can inhibit tumor growth by reducing the mobilization of proangiogenic BMDCs, thereby attenuating low-dose GEM's effect on promoting tumor growth.

It should be noted that Gr-1⁺CD11b⁺ is also a marker for myeloid-derived suppressor cells, which represent a heterogeneous population of immature myeloid cells capable of modulating immune responses (48, 49). Myeloid-derived suppressor cells are mobilized and recruited to tumor tissues to aid in establishing an immunosuppressive tumor microenvironment that makes it easier for tumor cells to avoid immunological detection (50, 51). This means that the promotion of tumor development and growth by GEM and/or CDDP might also be associated with immunosuppression of the tumor microenvironment, further research is needed into this.

Antineoplastic agents are usually applied in combination chemotherapy to increase therapeutic effect and reduce the toxic side effects (52, 53). GEM as a cell-cycle-specific drug that is usually combined with the nonspecific cell-cycle drug CDDP administration in breast cancer, to achieve a synergistic effect (54). The data obtained in the present study have shown that the promotion of tumor development and growth by GEM under continuous low-dose administration was also presented or even enhanced in the GEM + CDDP group *in vivo*. The enhancement of tumor cell functions such as proliferation, migration, and invasion were found in the GEM groups, and further amplified in the GEM + CDDP group *in vitro*. In addition, low concentrations of GEM + CDDP resulted in the increased mobilization of proangiogenic BMDCs and expression of proangiogenic protein, decreased proapoptotic factors, and increased the expression levels of antiapoptotic factors, resulting in the enhancement of angiogenesis and inhibition of tumor-cell apoptosis. Thus, combining low-dose antineoplastic agents might still result in similar or even greater facilitation of tumor formation or growth compared with when GEM is used alone. This aspect needs to be further explored due to its potential for widespread clinical applications.

Conclusions

Together, the present findings suggest that GEM or GEM + CDDP can facilitate tumor development and growth at low doses *in vivo* by promoting tumor-cell functions and inhibiting tumor-cell apoptosis directly, and by enhancing angiogenesis through the mobilization of proangiogenic cells in to tumor tissues indirectly. These results indicate the potential risks of the current empirical continuous low-dose chemotherapy regimens, and have revealed a preliminary pharmacological mechanism of action for explaining how antineoplastic agents at low dosages can promote tumorigenesis as well as occupational injuries to healthcare workers.

Abbreviations

ATRA: All-trans retinoic acid

BMDCs: Bone marrow derived cells

CD11b: Cluster of differentiation molecule 11b

CDDP: Cisplatin

DMSO: Dimethyl sulfoxide

GEM: Gemcitabine

MMP-9: Matrix metalloproteinase-9

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MVA: Microvascular area

PBS: Phosphate sodium buffer

VEGFR₁ Vascular endothelial growth factor receptor 1

VEGFR₂ Vascular endothelial growth factor receptor 2

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Xi'an Jiaotong University.

Consent for publication

The authors declare that they agree to submit the article for publication.

Availability of data and material

All data are included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

YSC, HL, QWZ and HLL performed the experiments; HNY, YF and KC provided material support; WBM, YCZ, JRK and ML commented on the study; YSC and WYF wrote the paper; YSC and WYF designed and supervised the research. All authors read and approved the final manuscript.

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Figures

Figure 1

Effects of continuous low-dose administration of GEM and/or CDDP once every 2 days on tumor progression and formation in mice. a-b-c B16 tumor volumes and weights 2 weeks after subcutaneous xenografts in C57BL/6J mice (n=7~8). d MCF-7 tumor weights and tumor formations 6 weeks after subcutaneous xenografts in nude mice (n=6). Data are mean $\pm$ SD values. *, $p < 0.05$; **, $p < 0.01$ vs control.

Figure 2

Effects of low-concentration GEM and GEM+CDDP on B16, MCF-7 and T-47D cell functions in vitro. a-b-c Effects of GEM and/or CDDP on the proliferation of MCF-7, T-47D, B16 cells. d Colony-formation assays in B16 cells treated with GEM and GEM+CDDP. Scale bar, 100 μ m. e Wound-healing assays in B16 cells treated with GEM and GEM+CDDP. Scale bar, 100 μ m. f-g-h Migration and invasion of MCF-7, T-47D and B16 cells treated with GEM and GEM+CDDP. Scale bar, 100 μ m. i-j-k Effects of GEM and GEM+CDDP on the cell cycle of MCF-7, T-47D, B16 cells. *, $p < 0.05$; **, $p < 0.01$.

Figure 3

Effects of low-concentration GEM and GEM+CDDP on apoptosis in B16, MCF-7, and T-47D cells. a-b-c B16 cell, MCF-7 cell and T-47D cell apoptosis were determined by flow cytometry analysis using Annexin V and PI staining. d-e Western Blotting to analyze the expression of apoptosis-related proteins in MCF-7 and T-47D cells. *, $p < 0.05$; **, $p < 0.01$.

Figure 4

Effects of low-dose GEM and GEM+CDDP on tumor angiogenesis in vivo and endothelial cells proliferation in vitro. a Effects of GEM and GEM + CDDP on micro-vessel of B16 (CD31+). Scale bar,100 μm . b Effects of GEM and GEM + CDDP on micro-vessel of B16 (Laminin). Scale bar,100 μm . c Effects of low concentration GEM and /or CDDP on the proliferation of endothelial cells. *, $p < 0.05$; **, $p < 0.01$.

Figure 5

Effects of continuous low-dose administration of GEM and GEM+CDDP once every 2 days on mobilization of Gr-1+CD11b+ and CD61+BMDCs in circulating blood of mice. a Gr-1+ CD11b+BMDCs counts in the circulating blood of MCF-7 tumor bearing nude mice treated with GEM and/or CDDP. b-c Gr-1+CD11b+BMDCs and CD61+BMDCs counts in the circulating blood of B16 tumor bearing C57BL/6J mice treated with GEM for 2 weeks. d-e Gr-1+CD11b+BMDCs and CD61+BMDCs counts in the circulating

blood of B16 tumor bearing C57BL/6J mice treated with GEM+CDDP for 2 weeks. f B16 tumor volume and weight of subcutaneous xenografts in mice with bone-marrow transplantation and GFP+ BMDCS in tumor tissue. Scale bar,100 μ m. *, p < 0.05; **, p < 0.01.

Figure 6

Effects of continuous low-dose administration of GEM and GEM+CDDP once every 2 days for 4 weeks on recruitment of GFP+BMDCs in B16 tumor tissues of C57BL/6J mice with GFP+ bone marrow. a B16 tumor volume and weight of subcutaneous xenografts in mice with bone-marrow transplantation. b GFP+ BMDCS in tumor tissue. Scale bar,100 μ m. *, p < 0.05; **, p < 0.01 vs control.

Figure 7

Effects of continuous low-dose administration of GEM and GEM+CDDP once every 2 days for 2 weeks on expression of proangiogenic factor proteins in C57BL/6J mice B16 tumor tissues. a-b The expression levels of pro-angiogenic proteins, including VE-cadherin, MMP-9, VEGFR1, and VEGFR2. *, $p < 0.05$; **, $p < 0.01$.

Figure 8

Effects of ATRA on the enhancement of B16 tumor growth and mobilization of proangiogenic BMDCs induced by continuous low-dose administration of GEM in C57BL/6J mice. a B16 tumor weights and volumes in C57BL/6J mice. b Gr-1+CD11b+ and CD61+BMDCs counts in the circulating blood of C57BL/6J mice treated with GEM and GEM combined with ATRA. c Effects of GEM and GEM combined with ATRA on micro-vessel of B16 (Laminin). Scale bar, 100μm*, p < 0.05; **, p < 0.01.