Antiangiogenic Effect of Cyclophosphamide by Metronomic Chemotherapy

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Abstract: Objective. To observe the effect of metronomic chemotherapy with cyclophosphamide (CTX) on tumor growth and angiogenesis on mouse Lewis lung carcinoma (LLC) model. **Methods.** LLC cells were injected subcutaneously into C57BL/6 mice on day 0. Mice were randomly divided into metronomic group, MTD group and control group. Therapies were given to the groups from the 6th day. Mice weight and tumor diameter were measured every other day. Then tumors were weighed. Microvessel count and proliferating index of tumor cell were performed by immunohistochemical staining with anti-CD_{31} antibody and anti-PCNA antibody. The histological characteristics of the tumor were detected by microscopy. **Results.** Tumor growth and angiogenesis were inhibited by CTX significantly in the metronomic group, which has better efficacy and lower toxicity than maximum tolerated dose (MTD) group. The average weight of the tumors in control group, metronomic group and MTD group were (3.72 ± 0.60) g, (1.85 ± 0.38) g and (1.73 ± 0.41) g, the average microvessel count were 26.38 ± 2.56, 15.09 ± 3.03 and 23.51 ± 2.78, PCNA index were 83.88 ± 3.72, 81.60 ± 4.21 and 71.80 ± 3.86, respectively. Necrosis was increased significantly in the metronomic group. **Conclusion.** CTX inhibits tumor growth and angiogenesis in mouse LLC model significantly. The inhibition may be correlated with the anti-angiogenic effect of CTX. [Life Science Journal. 2006;3(2):41-44] (ISSN: 1097-8135).

Keywords: metronomic chemotherapy; Lewis lung carcinoma; anti-angiogenesis

Abbreviations: CTX: cyclophosphamide; LLC: Lewis lung carcinoma; MTD: maximum tolerated dose

1 Introduction

One of the great advances in cancer treatment during last half of the 20th century was the development and utilization of chemotherapeutic drugs. These compounds have demonstrated anti-tumor efficacy through their damaging action against cellular DNA, which prevents proliferation and, frequently, drives the cell to its death. Since such effect is only exerted on a fraction of tumor cells, the administration of higher drug doses are required to achieve better clinical results. However, the application of the highest drug doses tolerated by the patient brings about the problem of drug toxicity. Thus, it is mandatory to establish rest periods, which not only allow regrowth of tumor cells but also growth of selected therapy-resistant clones. After the successful first cycles of treatment, tumors acquire resistance to the chemotherapeutic drugs. Thus, the growth of those resistant cells involve the development of more aggressive and malignant tumors.

In order to avoid the problems caused by traditional chemotherapeutic treatments, several researchers, recently began to search for new modalities of drug administration oriented towards a more efficient and non-toxic antitumoral and/or antimetastatic therapy. They found that some cytotoxic drugs, when given in low dose, high frequent mode, they could significantly inhibit angiogenesis in the tumor bed, resulting in preventing tumor growth because of ischemia. To be distinguished from traditional MTD, they called it metronomic chemotherapy[1], and its therapy target is endothelial cells.

The objective of this study was to investigate the antiangiogenic effect of cyclophosphamide (CTX) with metronomic chemotherapy on LLC mouse models, and to determine the toxicity of the treatment.

2 Materials and Methods

2.1 Animals, reagents and tumor specimens

30 male C57BL/6 mice (provided by Henan Experimental Animal Center) were used, weighing from 18 g to 22 g. CTX, in the injection power form, was the production of Jiangsu Hengrui Medical Corporation. LLC cells were preserved in C57BL/6 mouse and provided by Henan Medical

Science Institute.

2.2 Experimental models and therapeutic schedules

LLC cells were injected subcutaneously into the right oxter of C57BL/6 at 1×10^6/0.2 mL on day 0, and distributed randomly as follows: group Ⅰ control mice ($n=10$) were injected i.p. with saline once per day from day 6 until animals were killed; group Ⅱ metro group mice ($n=10$) were injected i.p. with CTX (20 mg/kg body weight) once per day, from day 6 until animals were killed; and group Ⅲ MTD group mice were injected i.p. with CTX (150 mg/kg body weight) once every other day over 6 days from day 6, followed by 2 weeks interval (21-day was a cycle).

Tumors were measured every other day from day 6 by a caliper. Tumor volumes were calculated as follows: $V=0.52\ (ab^2)$, where V = volume (mm^3), a = largest diameter (mm) and b = small diameter (mm) perpendicular with a. Animals were weighed every other day from day 6. On day 20, all animals were killed and tumors were excised. Tumors were weighed, fixed in 10% formalin and processed for immunohistochemical analysis. Paraffin blocks were cut to 4 μm sections and stained with hematoxylin and eosin (HE) for histological examination and with anti-CD_{31} and anti-PCNA antibody for assessment of microvessel density and tumor cell proliferation index, respectively.

2.3 Statistical analysis

We used statistical software package SPSS 12.0 to analyze the experiment results. The level of significance was set at $P<0.05$.

3 Results

3.1 Tumor growth assessment

Compared with control group, significant growth delays of tumor were observed in metro group, while in MTD group, the tumor volume decreased immediately after therapy, but increased during the rest periods. The growth curve was shown in Figure 1.

3.2 Body weight assessment

As for the body weight, steady increases were showed in control group and metro group, on the contrary, there were weight loss in the MTD group after therapy and weight increase during the rest periods. The results were showed in Figure 2. After mice were killed and tumors were excised, tumor weight in control group, metro group and MTD group were (3.72±0.60) g, (1.85±0.38) g and (1.73±0.41) g, respectively. Statistical differences were shown between control group and the other two groups.

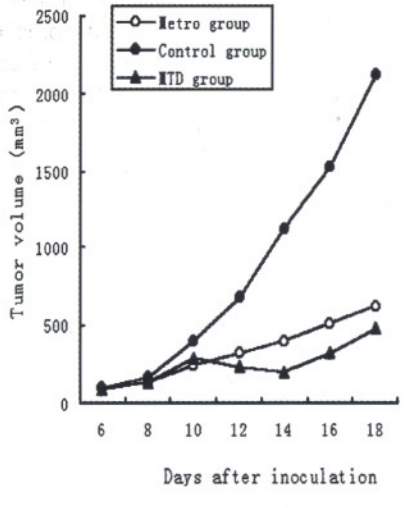

Figure 1. Curves of tumor volume

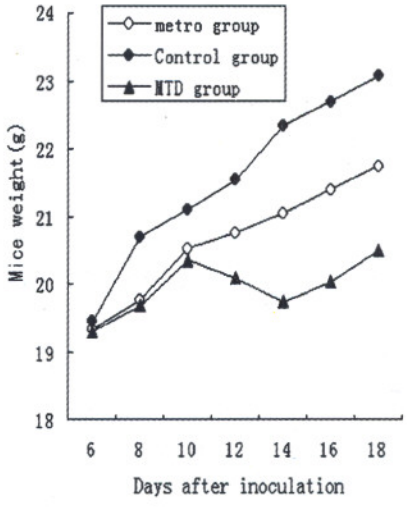

Figure 2. Curves of mice weight

3.3 Immunohistochemical results (Table 1)

On histological examination, no significant difference in morphology was found between tumors of the control group and therapy groups, but there were many necrosis areas in the metro group. Measurement of microvessel count and PCNA index showed differences among the groups of treatment. Tumors belonging to animals of the control group

showed the highest microvessel count and PCNA index. Those from animals submitted to MTD chemotherapy had intermediate microvessel count and the lowest PCNA index, whereas the lowest microvessel counts and intermediate PCNA index were measured in tumors from animals received metronomic schedule.

Table 1. Results of immuneohistochemical($\bar{\chi} \pm S$)

Group	Cases	Microvessel count	PCNA index
Control group	10	26.38 ± 2.56	83.88 ± 3.72
Metro group	10	$15.09 \pm 3.03^*$	81.60 ± 4.21
MTD group	10	23.51 ± 2.78	$71.80 \pm 3.86^*$

Note: $^*P<0.01$ compared to control group

4 Discussion

Historically, chemotherapy regimens have been controversial: which way should the scale be tipped between efficacy in tumor killing and lack of toxicity? On one hand, chemotherapeutic drugs could disrupt the DNA of tumor cells, breaking their replication and finally killing them, the befitting corollary being "the higher the dose the better"[2]. On the other hand, toxicity is found at several organ sites, which not only diminishes quality of patient life but also conspires against a good resolution of the cancer treatment, adding illness to that which already exists[3]. The introduction of maximum tolerated doses in usual treatment protocols made it necessary to impose intervals between cycles of therapy. During such intervals, recovery of "good" cells is frequently accompanied by recovery of "bad" cells, i.e. tumour cells resistant to the drug-resistant tumor cells. The possibility of finding a treatment modality that avoids toxicity without diminishing effectiveness is still a matter for study and discussion. The experimental findings of Browder[4], Klement[5,6] and Man[7] and colleagues successfully introduced a novel strategy for cancer treatment. The novel component comprised a cell target switch, which now aims towards the genetically stable tumor endothelial cells, along with a change in the schedule and dose of drug administration, thereby introducing metronomic chemotherapy[1].

The experimental models were designed to resemble the clinical situation of a patient with a recently detected tumor (not too large, but large enough to be easily detected) who begins therapy immediately after being diagnosed. Metronomic therapy began after 6 days of the tumor challenge. Our results showed that tumor growth and angiogenesis were significantly inhibited by low dose CTX. Tumor weight in metronomic group was statistically lower than that in control group. Body weight in metronomic and control groups increased steadily, which suggested that the toxicity of metronomic schedule was minimal. Though the short-term antitumor of MTD chemotherapy was effective, it accompanied with significant weight losses and tumor regrowth during the intervals. The lowest microvessel count and the lowest PCNA index were in metro group and MTD group, respectively. So we supposed that MTD schedule produced the effect mainly through inhibiting tumor cells growth or inducing cells death; while metronomic therapy induced tumor cells ischemia and necrosis mainly through damaging vessels in the tumor bed and its antitumor effect was related with its ability of decreasing microvessel count.

Other authors have demonstrated clearly the antiangiogenic nature of several drugs administered by metronomic dosing. Moreover, the combination of such treatment with specific antiangiogenic reagents increased significantly the observed antitumour effect of metronomic dosing[4-6,8]. In fact, literature dating back to the mid-1980s showed that virtually almost all the traditional chemotherapies had antiangiogenic effects or antivascular effects in various *in vitro* and *in vivo* assays[9]. But the antiangiogenic effects of chemotherapy were masked and marginalized by the general chemotherapy. In this case, the long intervals between drug administrations were necessary for the patient to recover from the harmful side effects of both the MTD and the anti-angiogenic chemotherapy drugs. So, if we give drugs more frequently, such as once or more per week without extended breaks, there would be significantly less opportunity for repairing the damaged endothelium and the anti-angiogenic effects of the chemotherapy. This, of course, necessitates reducing the dosage of the drug administered with each injection.

In short, metronomic chemotherapy has many advantages, but its molecular mechanism is unclear and there are several significant challenges in clinical applications. Foremost among these is the current empiricism associated with determining the optimal dose and schedule for administration of chemotherapeutics. A second challenge is the prospect of delayed side effects, including secondary neoplasms. In particular, it should be clear about which chemotherapeutics are the most effective for metronomic regimens, what kind of combinations and sequences might be the best, and what mechanisms of resistance might develop for a long-term therapy.

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Received December 25, 2005