Low-dose metronomic chemotherapy of paclitaxel synergizes with cetuximab to suppress human colon cancer xenografts

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Low-dose metronomic (LDM) chemotherapy represents a new strategy to treat solid tumors by stronger antiangiogenic activity and lower side effects, especially in combination with other antiangiogenic agents. This study aims to investigate whether LDM chemotherapy of paclitaxel could synergize with cetuximab, an antiangiogenic agent to suppress HT-29 human colon tumors in BALB/c nude mice. To explore its possible mechanism, the tumor vascular status was detected by staining with anti-CD31 Ab and the tumoral expression of thrombospondin-1 was examined by immunohistochemistry, western blot analysis, and real-time PCR. Our results showed that empirical metronomic paclitaxel regimens in combination with cetuximab induces significant and durable antitumor responses without overt toxicity. Paclitaxel LDM chemotherapy displayed stronger antiangiogenic activity than maximum tolerable dose (MTD) chemotherapy, whereas MTD chemotherapy induced more apoptotic cells. The combinational therapy with LDM and cetuximab showed the strongest antiangiogenic activity among all the groups. Paclitaxel LDM chemotherapy also dramatically upregulated the expression of thrombospondin-1, but MTD chemotherapy did not. These results suggest that the combination of paclitaxel LDM chemotherapy and cetuximab represents a potent strategy to combat colon cancers. *Anti-Cancer Drugs* 20:355–363 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide [1]. Surgery remains the mainstay of treatment for colorectal cancer, but it is ineffective for distant metastases [2]. Despite extensive exploration for new anticancer drugs or therapeutic strategies, the success of current treatments is still unsatisfactory, and the side effects of chemotherapy can be particularly harsh. Recently, there has been considerable interest in the notion of exploiting chemotherapeutic drugs as angiogenesis inhibitors [3,4]. The mode of action is thought to be explained by the interference of these agents with cycling and proliferating endothelial cells in the process of tumor angiogenesis [5]. Conventional application of chemotherapy, targeting the tumor cells directly, is based on the linear dose–efficacy relationship for these drugs resulting in a cyclic treatment to allow for recovery from the side effects. Any potential damage to the tumor vasculature can be repaired during the long breaks. Continuous, low-dose chemotherapy, that is, metronomic chemotherapy (LDM), targeting the endothelial cells directly is thought to have fewer side effects as well as a lack of drug resistance [6,7]. Paclitaxel seems to be a strong candidate for LDM chemotherapy given its ability to inhibit endothelial cell functions relevant to angiogenesis *in vitro* at extraordinarily low concentrations and broad-spectrum antitumor activity [8]. The effects of LDM chemotherapy regimens can also be improved by concurrent administration of a drug-targeting angiogenesis [9,10]. The rationale for this combination is that these targeted drugs capable of specific blockade of activated endothelial cell survival mechanisms, will selectively enhance the damaging or cytotoxic effects of LDM chemotherapy on newly formed blood vessels [11]. The epidermal growth factor receptor (EGFR) and its ligands EGF and tumor growth factor-α play important roles in the growth and survival of colorectal tumors [12]. The expression of EGFR in CRC correlates with more aggressive diseases and poor prognosis. Hence, blockade of the EGFR is a potent strategy for treatment of colorectal cancer. Cetuximab, an IgG_1 monoclonal antibody that binds the EGFR with high affinity, competes for ligand binding, and downregulates receptor expression on the cell surface [13,14]. Several studies have shown that cetuximab inhibited the proliferation of EGFR-expressing tumor cells *in vitro*, and markedly suppressed human colorectal tumors established in nude mice [15]. Cetuximab displays antiangiogenic activity by downregulating the angiogenic factors vascular endothelial growth factor, IL-8, and basic fibroblast growth factor, which are expressed by the tumor cells [16–18]. Therefore, we designed this study to investigate the efficacy of LDM chemotherapy with paclitaxel, alone or in combination with cetuximab, in

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a nude mouse model of HT-29 human colon carcinoma tumors.

Materials and methods

Cell culture

Human colon carcinoma cell lines HT-29, HCT-116, RKO, and SW620 were provided by the Institute of Biochemistry and Cell Biology of China (Shanghai, China), and the human umbilical vein endothelial cells (HUVECs) were supplied from the Typical Animal Reserve Center of China. The cells were routinely cultured in RPMI 1640 medium supplemented with 550 U/ml of penicillin/streptomycin, 2 mmol/l of L-glutamine and 1 mmol/l pyruvate, at 37°C in a humidified atmosphere containing 5% CO_2. Tumor cell suspensions of greater than 90% viability were prepared from subconfluent cultures with 0.25% Trypsin and 0.02% EDTA. Trypan blue exclusion was performed to ensure cell viability.

MTT assay

Cells (4×10^3) were seeded in 200 µl of RPMI 1640 into 96-well plates. The medium was replaced 12 h later with 200 µl of fresh RPMI 1640 media containing 0.00036, 0.0036, 0.036, 0.36, 3.6, 36 or 360 µg/ml of paclitaxel. The cells were cultured for a further 48 h. Cells cultured in 200 µl of RPMI 1640 served as controls. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (20 µl) was added to each well followed by a 4-h incubation, and cells were processed to record the optical density (OD) value at 490 nm. The proliferation inhibition rate (%) was calculated according to the formula: (1–experimental OD value/control OD value) × 100%. The experiments were repeated thrice.

Mice and tumor cell inoculation

Female 4–5-week-old BALB/c nude mice were obtained from the Experimental Animal Center of the First Clinical Medical School of Harbin Medical University, China. All surgical procedures and care administered to the animals were in accordance with institutional guidelines. After a 2-week quarantine, mice were included in the protocols. HT-29 cells (2×10^5) in 0.2 ml of medium were subcutaneously injected into the right flank of the mice. Tumor volumes were estimated according to the formula: $\pi/6 \times a^2 \times b$, where a is the short axis, and b is the long axis. Approximately 2 weeks later (average tumor size, 100 mm^3), the mice were randomized into five groups (each group had 12 mice) and treated for 8 weeks as follows: control (i.p. injection of 0.2 ml physiological saline daily); maximum tolerable dose chemotherapy (MTD) [intraperitoneal (i.p.) injection of 20 mg/kg paclitaxel (Taxol, Bristol-Myers Squibb Company, Germany) once a week]; LDM (i.p. injection of 1.5 mg/kg paclitaxel daily); cetuximab (i.p. injection of 0.5 mg cetuximab every 3 days) and the combinational therapy with LDM paclitaxel and cetuximab at doses identical to the single agent. The doses of paclitaxel and cetuximab were chosen according to previous studies [8,19]. The mice were closely monitored and body weight and tumor size recorded twice a week. Blood samples were collected through tail vein once a week to count white blood cells. Moribund mice were euthanized according to the preestablished criteria, namely the presence of one or more of the following premorbid conditions: gross ascites, palpable tumor burden greater than 2.0 cm^3, dehydration, lethargy, or weight loss greater than 20% of initial body weight. When the size of tumors in the control group reached to 2.0 cm^3 42 days after implantation, all the mice in this group were killed, and five mice were randomly killed from each other group, tumors and eyes were removed.

Assessment of retinal vascular pattern

To assess the retinal vascular patterns using the trypsin digestion method described previously [20], the eyes were fixed in 4% paraformaldehyde after enucleation, and the retinas were removed from the eyecup and washed. After incubation at 37°C in 3% trypsin solution for 3 h, the digested retinas were transferred to PBS and the internal limiting membrane was peeled. The vascular frame was isolated from the retinal background with glass sticks and mounted on a slide glass dried and stained with hematoxylin and eosin to visualize the retinal vascularity.

Histology

Tumor specimens were fixed in 10% buffered formalin for 24 h, transferred to 70% ethanol, and subsequently paraffin-embedded and sectioned. Tumor sections were rinsed with PBS, blocked with 3% BSA for 2 h, and incubated overnight with primary antibodies. They were subsequently incubated for 30 min with appropriate secondary antibodies using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), and immunoreactivity developed with Sigma FAST DAB (3,3′-diaminobenzidine tetrahydrochloride) and $CoCl_2$ enhancer tablets (Sigma-Aldrich, Shanghai, China). General tissue morphology and mitotic figures were visualized by hematoxylin and eosin staining. Tumor microvessels and thrombospondin (TSP)-1 expression were visualized using immunofluorescence detection of CD31, and TSP-1, respectively. Stained vessels were counted in 10 blindly chosen random fields at × 200 magnification, and the mean microvessel density was recorded.

TUNEL assay

The methodology has been described previously [21]. In brief, tumor sections were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) agent (Roche, Shanghai, China), and examined by microscopy. TUNEL labeling was assessed on two sections (> 50 µm apart) per tumor and 5–10 randomly chosen fields (×400) per section.

Quantitative real-time PCR assay

Real-time quantitative reverse transcription (RT) PCR was performed using ABI PRISM 7900 (Applied Biosystems, Applera Corporation, USA). RT-PCR amplification mixture contained template cDNA, 2 × TaqMan master mix (Applied Biosystems) and primers for TSP-1 (5′-CACGCTACAGGACAGCAT-3′, 5′-GGCCGCCTCA GCTCATT-3′). RT-PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative mRNA quantification was performed using the $\Delta\Delta C_T$ method normalized to β-actin and expressed as a fold difference compared with control tumors.

Western blot analysis

Cells sonicated in RIPS buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethysulfonyl fluoride, 45 μg/ml Aprotinin, 100 mmol/l sodium orthovanadate) comprised 5×10^5 and homogenized. All the tumor tissues were excised, minced, and homogenized in protein lysate buffer as described previously. Debris was removed by centrifugation at 10 000*g* for 10 min at 4°C. The content of protein in the homogenates from cells or tumor tissues was determined, samples containing 20 μg of total protein were resolved on 12% polyacrylamide SDS gels, and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, incubated with primary antibodies, and subsequently with alkaline phosphatase-conjugated secondary antibody. They were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co. Ltd., Beijing, China). Blots were stained with anti-β-actin Ab to confirm that each lane contained similar amounts of tumor homogenate.

Statistical analysis

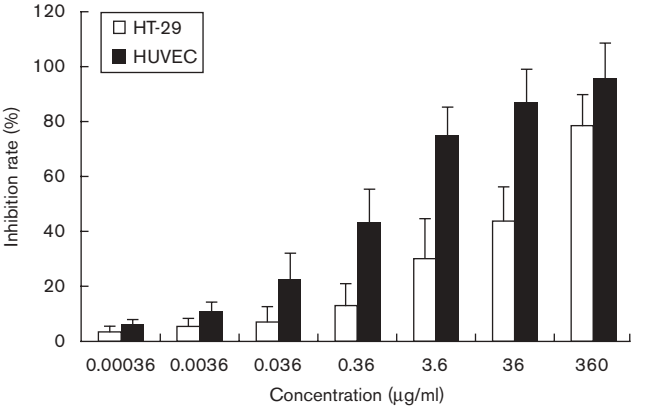
Results are reported as means ± SD. Statistical significance of differences was assessed by analysis of variance, followed by the Student–Newman–Keuls test, using SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). The level of significance was set at a P value of less than 0.05.

Results

Paclitaxel inhibits proliferation of HUVEC and HT-29 cells

We demonstrated whether paclitaxel inhibits the proliferation of HUVECs and HT-29 cells *in vitro* with MTT assay. As shown in Fig. 1, paclitaxel inhibited the proliferation of the cells incubated with paclitaxel for 48 h in a dose-dependent manner, compared with untreated cells. At the concentration of 3.6 μg/ml, paclitaxel was able to almost completely arrest the proliferation of HUVECs, but only inhibited the proliferation of HT-29 cells by a rate of 30.2% compared with untreated cells. At the concentration of 360 μg/ml, paclitaxel could almost arrest the proliferation of both HUVECs and HT-29 cells (Fig. 1). With a simple linear regression analysis, the half maximal inhibitory concentration (IC_{50}) by paclitaxel was calculated to be 1.78 μg/ml for HUVECs, but the IC_{50} for CT-26 cells was 182.84 μg/ml.

Fig. 1

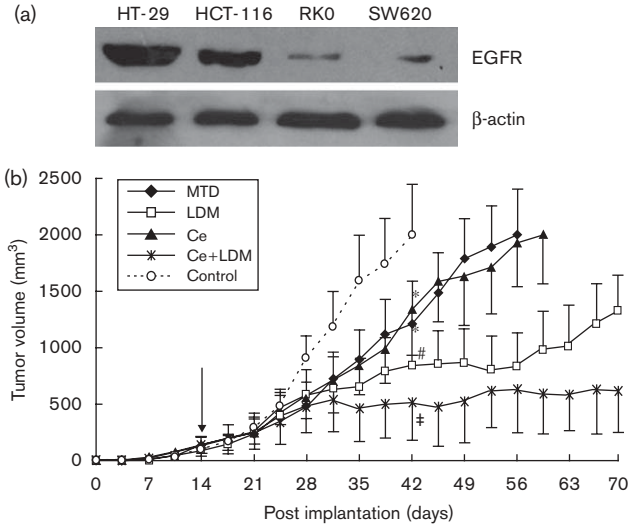


Paclitaxel inhibits the proliferation of HT-29 cell and human umbilical vein endothelial cell (HUVEC) *in vitro*. The cells were incubated in the absence or presence of paclitaxel at different concentrations, and harvested 48 h later. The proliferation of cells was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method to calculate the proliferation inhibition rate (%).

Paclitaxel LDM chemotherapy synergizes with cetuximab suppresses the growth of HT-29 tumors

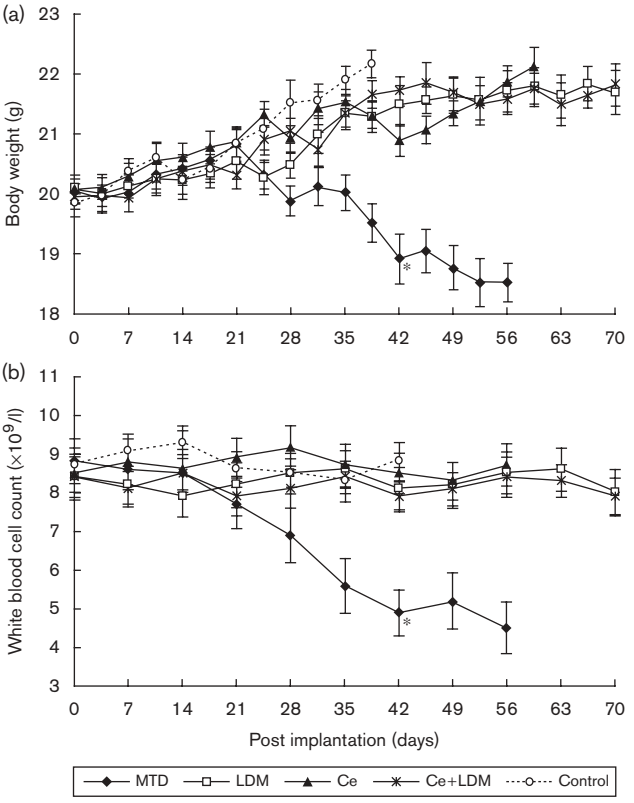
First, we detected the expression of EGFR in HT-29 cells as well as other three colon cancer cells. As shown in Fig. 2a, EGFR was shown to be strongly expressed in both HT-29 and HCT-116 cells, but weakly expressed in RKO and SW620 cells. Moreover, it is now well recognized that EGFR antibody blockade effectiveness is dependent upon K-ras status [22,23], and Hamada *et al.* [24] reported that no K-ras mutations were observed in HT-29 cancer cell lines, which provided the rationale for the use of cetuximab to treat HT-29 tumors. Then, tumors were established by subcutaneous injection of HT-29 tumor cells into the mice. Two weeks later, when the size of tumors reached around 100 mm^3, the mice were randomly assigned to five groups as stated in Materials and methods. As shown in Fig. 2b, the tumors in the control group grew rapidly, reaching 2000 mm^3 in volume 42 days after implantation; thus all the mice in this group were killed according to the preestablished criteria. In contrast, in the MTD and cetuximab groups, the tumors had reached only 1209 ± 377 mm^3 and 1346 ± 412 mm^3 42 days after implantation, respectively, being significantly smaller than the control tumors (both $P < 0.01$). Furthermore, the tumors in the LDM group was significantly smaller than that in the MTD group ($P < 0.05$), and the combination of paclitaxel and

Fig. 2



Paclitaxel low-dose metronomic (LDM) chemotherapy synergizes with cetuximab to suppress the growth of epidermal growth factor receptor (EGFR)-positive HT-29 cells *in vivo* and prolongs the survival of tumor-bearing mice. (a) Western blot analysis of EGFR expression in homogenates of four types of human colon cancer cells. (b) HT-29 colon tumors were established in BALB/c nude mice. Two weeks later, when the size of tumors reached around 100 mm^3 (indicated by a vertical arrow), they were assigned into five groups: control, LDM, maximum tolerable dose (MTD), cetuximab (Ce), and Ce+ LDM. A significant difference in tumor volumes of controls are $^*P=0.0263$ and 0.0295 of MTD and Ce groups, respectively and a highly significantly difference is $^{\#}P=0.0062$. Significant difference from LDM therapy is $^{\ddagger}P=0.0324$.

Fig. 3



Body weight and white blood cell counts. The mice were weighed (a) and blood collected at indicated time points to count white blood cells (b). A significant difference in body weight or white blood cell counts between the maximum tolerable dose (MTD) group and the other four groups at the indicated time point is denoted by '*'. Ce, cetuximab; LDM, low-dose metronomic.

cetuximab markedly suppresses the growth of tumors, resulting in smaller tumors than paclitaxel and cetuximab monotherapies (both $P < 0.01$), respectively.

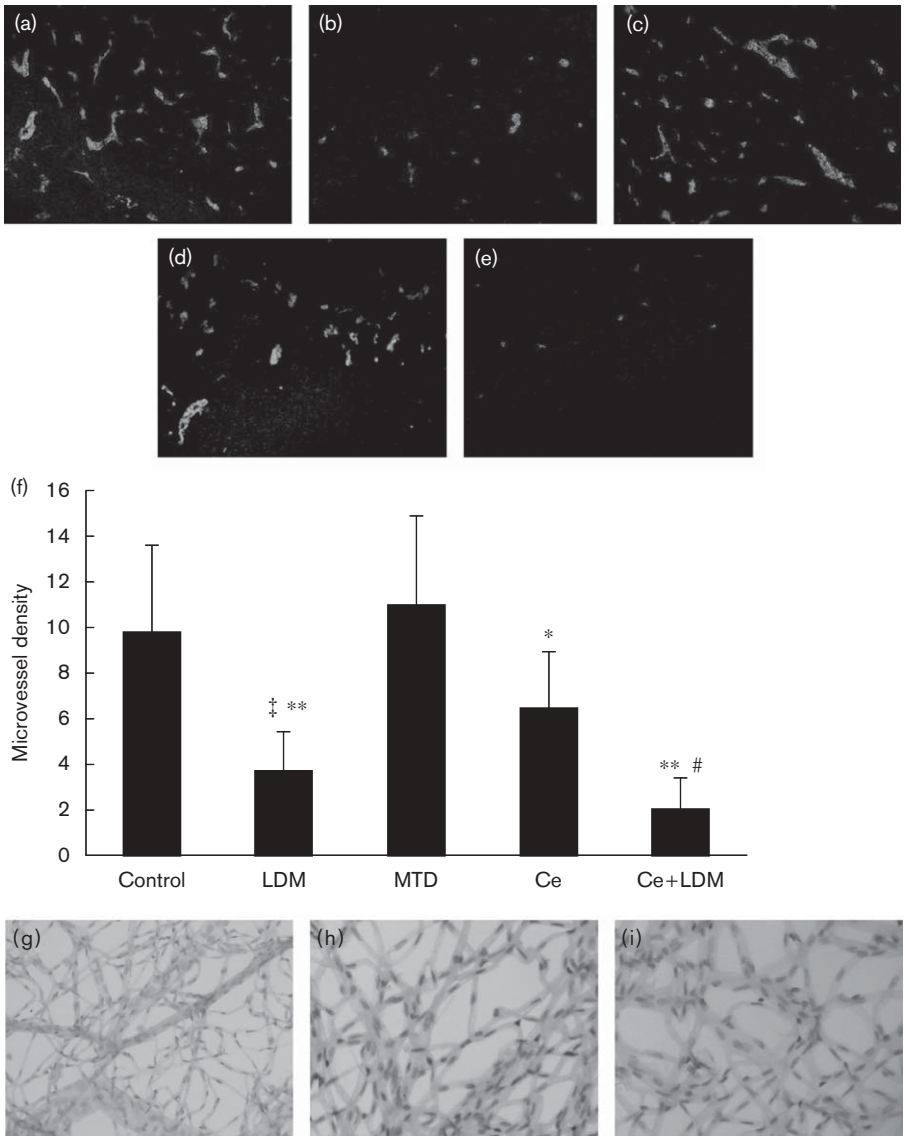
Paclitaxel LDM chemotherapy induces less side effects

As shown in Fig. 3a, the five groups of mice had a similar body weight in the beginning, but 2 weeks after initiation of antitumor therapy, an obvious decrease of animal weight was observed in the MTD group partially associated with diarrhea. Forty-two days after tumor implantation, the average body weight of mice in the MTD group was 18.9 ± 0.4g, which was significantly lower than that of the mice in the control (22.1 ± 0.3 g), LDM (21.5 ± 0.3 g), and the combinational (21.7 ± 0.5 g) groups (All $P < 0.05$). Similarly, as shown in Fig. 3b, a marked reduction of white blood cell count (WBC) counts was observed in mice of the MTD group. However, there were no significant differences in WBC counts in the other four groups.

Paclitaxel LDM chemotherapy inhibits formation of new microvessels but does not affect normal microvessels

The tumors mentioned above were harvested, sectioned, and stained with an anti-CD31 Ab to visualize microvessels. Representative photographs were taken for the mice in control (Fig. 4a), LDM (Fig. 4b), MTD (Fig. 4c), cetuximab (Fig. 4d) and the combination of LDM plus cetuximab groups (Fig. 4e). There were fewer microvessels in tumors in the LDM chemotherapy (Fig. 4b), compared with the control tumors (Fig. 4a), and the combinational therapy almost completely diminished the microvessels (Fig. 4e). Tumor microvessels in sections were counted in blindly chosen random fields to record microvessel density (Fig. 4f). Cetuximab treatment resulted in a statistically significant ($P < 0.05$) reduction by 33% in tumor microvessel density compared with the controls, whereas LDM treatment and the combinational therapy led to highly statistically significant ($P < 0.001$) 63 and 79% reduction in tumor microvessel density compared with the control, respectively. The mean microvessel density in mice treated with LDM was also significantly ($P < 0.05$) lower than that in the MTD group, indicating that LDM chemotherapy had a stronger antiangiogenic activity than MTD. The combinational therapy resulted in further reduction of the blood vessel density by 46% compared with that in the LDM group.

Fig. 4



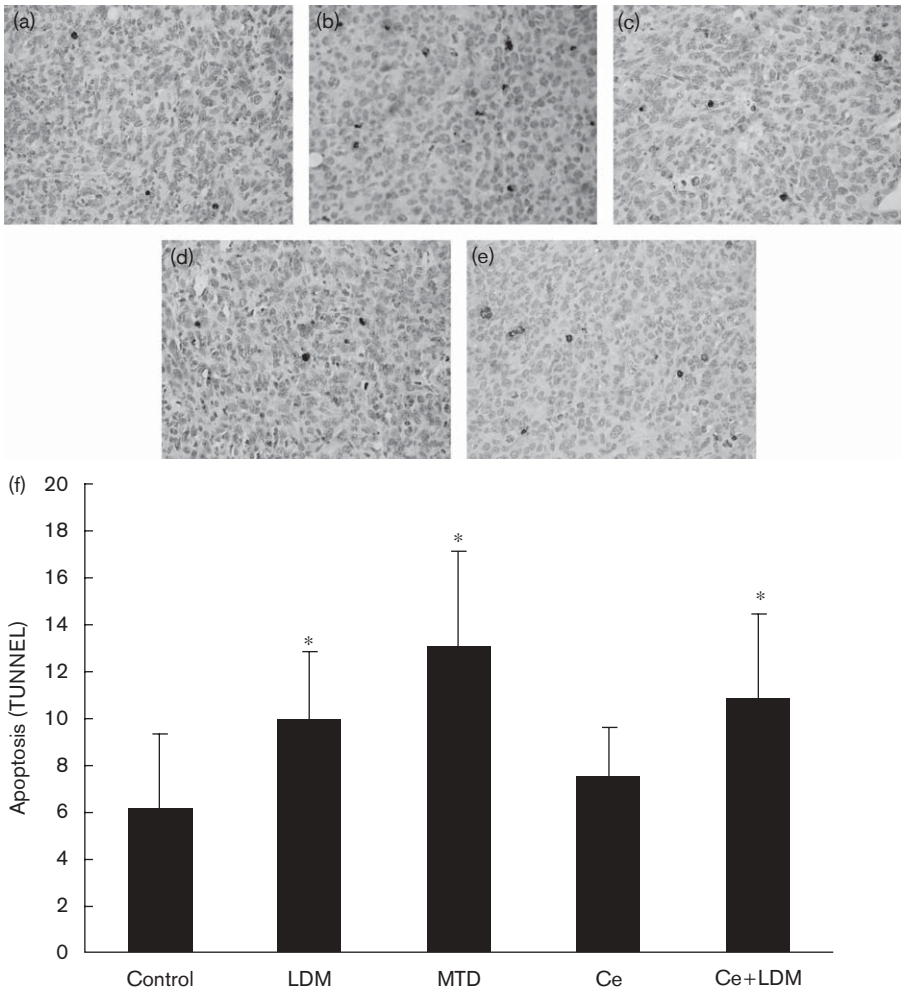
Paclitaxel low-dose metronomic (LDM) therapy synergizes with cetuximab to inhibit tumor angiogenesis and has no apparent effect on normal retinal vascularity. (a–e) Illustrated are representative tumor sections prepared from the mice in the control (a), LDM (b), maximum tolerable dose (MTD) (c), cetuximab (Ce) (d), and Ce + LDM (e) groups, 42 days after cell implantation in Fig. 2. Tumor microvessels in sections were immunofluorescence stained with the anti-CD31 Ab (× 200 magnification). (f) The microvessels were counted to record microvessel density. A significant difference in microvessel densities of controls is $*P=0.0381$ and a highly significantly difference is $**P=0.0074$ and 0.0042 of LDM and Ce + LDM groups, respectively. Highly significant difference from the MTD group is $^{\ddagger}P=0.0052$, and significant difference from the LDM group is $^{\#}P=0.0364$. (g–i) The retinas from the mice in the control (g), LDM (h), and MTD (i) groups were removed and stained with hematoxylin and eosin to view the retinal vascular architectures (× 200 magnification).

In addition, to further elucidate whether the LDM chemotherapy affects the growth of microvessels on other parts of the body apart from the tumor, we examined whether paclitaxel could affect the retinal vascular architecture. The results did not show any obvious difference in retinal vascular architecture among mice in the control (Fig. 4g), LDM (Fig. 4h), and MTD (Fig. 4i) groups.

Paclitaxel induces cell apoptosis *in situ*

The tumor sections from above were stained with TUNEL and examined under light microscopy. A small number of apoptotic cells were detected in the control tumors (Fig. 5a), whereas more apoptotic cells were detected in tumors treated with MTD (Fig. 5b), LDM (Fig. 5c), and the combination of LDM plus cetuximab (Fig. 5e). Cetuximab therapy did not induce more

Fig. 5



Paclitaxel low-dose metronomic (LDM) therapy synergizes with cetuximab to induce cell apoptosis *in situ*. (a–e) Illustrated are representative tumor sections prepared from the mice in the control (a), LDM (b), maximum tolerable dose (MTD) (c), cetuximab (Ce) (d) and Ce + LDM (e) groups, 42 days after cell implantation. Tumor sections were stained with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) to view apoptotic cells (× 200 magnification). (f) The TUNEL-positive cells were counted. A significant difference in counts of TUNEL-positive cells from controls is $*P=0.0402$, 0.0257, and 0.0315 of LDM, MTD, and Ce + LDM groups, respectively.

apoptotic cells compared with control (Fig. 5d), and the MTD treatment induced even more apoptotic cells than LDM therapy. The TUNEL positive cells of tumors treated with MTD, LDM, and the combinational therapy were significantly higher (all $P < 0.01$) than that of the control tumors by 112, 61, and 77%, respectively (Fig. 5f).

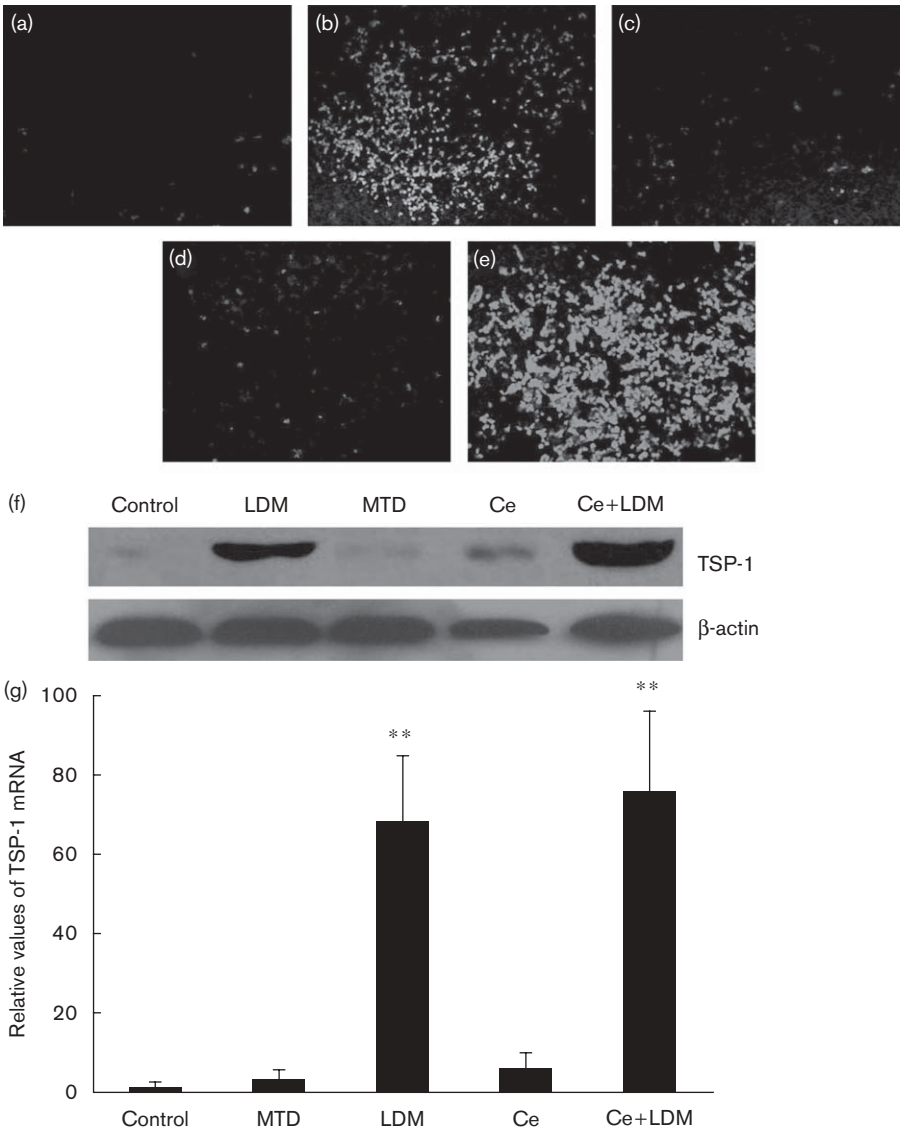
Expression of thrombospondin-1

TSP-1 has been regarded as a secondary mediator of the antiangiogenic effects of some LDM chemotherapy regimens. Therefore, we used multiple methods to detect the tumoral expression of TSP-1. Immunofluorescence analysis showed that TSP-1 expression was dramatically upregulated in the tumors from the LDM (Fig. 6b) and combinational therapy groups (Fig. 6e), compared with controls (Fig. 6a); whereas, MTD (Fig. 6c) and cetuximab (Fig. 6d) therapies did not increase its expression. The results were supported by western blot analysis of tumor homogenates (Fig. 6f), and further supported by mRNA expression detected by real-time PCR that the LDM and the combinational therapies highly significantly up-regulated TSP-1 mRNA (both $P < 0.001$), but MTD and cetuximab therapies only slightly increased TSP-1 mRNA expression, which was not significantly different from the controls (Fig. 6g).

Discussion

It has become apparent that tumor endothelial cells are sensitive to the action of conventional cytotoxic drugs, if the dosing regimen is altered [25,26]. The concept, known as LDM chemotherapy, was shown in preclinical studies using transplanted tumor models. The frequent

Fig. 6



Expression of thrombospondin (TSP)-1 in tumors. Illustrated are representative tumor sections prepared from the mice in the control (a), low-dose metronomic (LDM) (b), maximum tolerable dose (MTD)(c), cetuximab (Ce) (d) and Ce+ LDM (e) groups, 42 days after cell implantation in Fig. 2. Tumor sections were immunofluorescence stained with an anti-TSP-1 Ab (× 200 magnification). (f) Tumor homogenates were western blotted with anti-TSP-1 (upper panel), or β-actin (lower panel) antibodies. (g) Real-time quantitative reverse transcriptase-PCR analysis of TSP-1 mRNA expression in tumors above. The levels of TSP-1 mRNA were presented as relative values to control. A highly significant difference from controls is $^{**}P = 0.0018$ and 0.0012 of LDM and Ce+ LDM groups, respectively.

administration of comparatively low doses of cytotoxic agents, with no extended breaks, may not target tumor cells directly, as is primarily the case for the cyclic MTD approach, but indirectly through inhibiting angiogenesis and vasculogenesis. As such, LDM chemotherapy may offer several advantages over the MTD approach, including reduced toxicity, and treatment response irrespective of the resistance profile of the tumor cell population [26]. However, it is noteworthy that previous studies indicate that low-dose chemotherapy protocols sometimes were not effective on their own and, indeed, in some cases, actually stimulated tumor growth for reasons that are as yet unclear [27]. Hence, we use a combination of empirical metronomic paclitaxel regimens with an EGFR-blocking antibody, and these results show that this therapeutic schedule that induces significant and durable antitumor responses is encouraging.

Our study has added further support for the potential therapeutic value of LDM chemotherapy, and also suggested a strategy for enhancing its antiangiogenic efficacy by concurrent administration of cetuximab, an

EGFR-blocking antibody. These results have revealed several new and important aspects of continuous LDM chemotherapy combined with anti-EGFR-blocking antibodies. In the present research, paclitaxel had a significant decrease in IC_{50} values of endothelial cells compared with HT-29 tumor cells *in vitro*, indicating antiangiogenic doses are far below the optimal doses for direct antitumor activity. Furthermore, our study is the first to show the antitumoral and antiangiogenic activity of paclitaxel LDM chemotherapy in combination with cetuximab in the human HT-29 colon cancer xenografts. Moreover, we did not observe toxic effects at the doses administered in the LDM and combinational groups, as evidenced by body weight and WBC counts, which remained similar to those of control mice during the treatment period. To understand better whether paclitaxel metronomic therapy might affect the microvessel growth on other parts of the body apart from the tumor, we chose retinal digest preparations to observe the microvessel morphology, and no significant difference was found in the control group. These observations suggest the logic for integrating chemotherapy with other vascular-targeting drugs so as to improve efficacy while circumventing the acute undesirable toxic side effects associated with standard or high-dose chemotherapy.

Besides, recent retrospective data have shown a very negative association between K-ras mutations and cetuximab response [23,28]. Mutant K-ras occurs in approximately half of all CRC cases, and is associated with chemoresistance and radioresistance [29]. Pervious data showed that no K-ras mutations were observed in HT-29 cancer cell lines used in our study, but would the presence or absence of a K-ras mutation affect consideration for this combinational therapy? Goncalves *et al.* [30] reported that K-ras mutations do not preclude any cetuximab-based combination efficacy, and clinically relevant tumor responses and long-term stabilization may be achieved with cetuximab-based treatment in patients with K-ras mutated tumors. We believe that it could be premature to absolutely exclude cetuximab use in K-ras mutated tumors, and this conception needs further investigation.

Furthermore, previous studies showed that LDM chemotherapy alone sometimes resulted in transient tumor growth inhibition, increased hypoxia, and finally stimulated tumor growth [31]. It is reported that metronomic cisplatinum chemotherapy can cause such changes as an increase in endothelial cell VEGF or enhanced expression of tumor cell-associated EGFR, either of which could promote tumor growth. Moreover, Luwor *et al.* observed that cetuximab reduced level of hypoxia-inducible factor 1α (HIF-1α), which appears to act mainly by reducing the synthesis of the HIF-1α protein in both normoxic and hypoxic conditions [32]. The reduction of the level of HIF-1α was accompanied by transcriptional inhibition of VEGF expression, and these results may account for the synergistic effect of LDM chemotherapy and cetuximab observed in our study. To test this hypothesis, we are currently investigating the combinational therapy of LDM chemotherapy and HIF-1α knockdown in our ongoing research.

An obvious question raised by our results is why tumor vascular endothelial cells appear to be selectively sensitive to protracted exposure of low concentrations of chemotherapeutic drugs. It is possible that the inhibition of endothelial cell growth or induction of apoptosis may not be directly mediated by the chemotherapeutic drugs tested, but rather are secondary to an event induced by the drugs. One of these antiangiogenic effect mediators is TSP-1 [33]. Bocci *et al.* [34] showed that TSP-1 in the tumor microenvironment is a key mediator of antiangiogenic effects observed with low-dose antiangiogenic scheduling of cyclophosphamide. TSP-1 is a multifunctional protein and activated by tumor suppressor gene products such as p53. Moreover, TSP-1 is expressed by different cells, including perivascular cells, stroma cells, and platelets, and can inhibit endothelial cell growth [33]. In addition to these activities, the intact TSP-1 molecule, as well as specific peptide fragments, is able to induce apoptosis in endothelial cells [35]. Besides, another way by which LDM chemotherapy induces an antiangiogenic effect is by decreasing the viability of circulating bone marrow-derived endothelial precursor cells and therefore act in a similar way as other antiangiogenic agents, including endostatin and angiostatin [36,37]. This study has shown that paclitaxel LDM and combinational therapy significantly induced tumoral expression of TSP-1. However, the cellular mechanism for this induction of TSP-1 still awaits elucidation.

In conclusion, the results presented here have shown an effective and safer strategy by using paclitaxel LDM chemotherapy alone or by combining it with cetuximab, compared with paclitaxel MTD therapy in respect of tumor growth, animal survival, and side effects, in a nude mouse model of HT-29 human colon cancers. Although a potential major advantage, especially when integrated with targeted antiangiogenic drugs, several challenges must be overcome to realize the full benefits of this therapeutic approach in the clinic, such as defining the optimal low dose and schedule. Clinical trials, and further preclinical studies, will hopefully provide answers to these and similar questions surrounding the use of antiangiogenic therapies for the treatment of colon malignancies.

Acknowledgements

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