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Cytochalasin D, a tropical fungal metabolite, inhibits CT26 tumor growth and angiogenesis

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ABSTRACT

Objective: To investigate whether cytochalasin D can induce antitumor activities in a tumor model. **Methods:** Murine CT26 colorectal carcinoma cells were cultured *in vitro* and cytochalasin D was used as a cytotoxic agent to detect its capabilities of inhibiting CT26 cell proliferation and inducing cell apoptosis by MTT and a TUNEL–based apoptosis assay. Murine CT26 tumor model was established to observe the tumor growth and survival time. Tumor tissues were used to detect the microvessel density by immunohistochemistry. In addition, alginate encapsulated tumor cell assay was used to quantify the tumor angiogenesis *in vivo*. **Results:** Cytochalasin D inhibited CT26 tumor cell proliferation in time and dose dependent manner and induced significant CT26 cell apoptosis, which almost reached the level induced by the positive control nuclease. The optimum effective dose of cytochalasin D for *in vivo* therapy was about 50 mg/kg. Cytochalasin D *in vivo* treatment significantly inhibited tumor growth and prolonged the survival times in CT26 tumor–bearing mice. The results of immunohistochemistry analysis and alginate encapsulation assay indicated that the cytochalasin D could effectively inhibited tumor angiogenesis. **Conclusions:** Cytochalasin D inhibits CT26 tumor growth potentially through inhibition of cell proliferation, induction of cell apoptosis and suppression of tumor angiogenesis.

1. Introduction

Actin has been deeply studied and concluded as an evolutionary conserved molecule and one of the major cytoskeletal components in eukaryotic cells including tumor cells[1, 2]. Actin exists in two forms, the monomeric actin and microfilament that is a polymerized form of monomeric actin. Each microfilament has two different specific ends, at which polymerization happens at different rates, a fast growing or ‘barbed’ end and a slow–growing or ‘pointed’ end, which keep in a dynamic balance between

monomeric and polymerized actins[2–4]. The polymerization, depolymerization and redistribution of actin microfilaments are delicately regulated by a variety of extracellular and intracellular factors[1, 5, 6]. At present, more and more study results have indicated that microfilaments are involved in various cellular functions including cell morphology, migration, ion channels activity, secretion, apoptosis and cell survival. Therefore, disruption of microfilaments by chemical agents, such as cytochalasins, can cause severe damages in cell function, even culminating in cell death or apoptosis[7–10].

Cytochalasins are a group of chemical agents, which are mainly isolated from symbiotic fungi in tropical countries and areas. Cytochalasins are capable of permeating cell membranes, binding to actin and altering its polymerization. At present, several lines of evidence have indicated that cytochalasin B and E possess somewhat anticancer activities[11, 12]. However, cytochalasin D (Cyt D) is thought to be the most specific for the actin microfilaments among various cytochalasins at present[13]. Functionally, Cyt D

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can specifically binds to the barbed end of growing actin microfilaments and at last causes inhibition or disruption of actin microfilaments by altering actin polymerization[2]. Therefore, it is reasonable to consider that Cyt D may be a candidate cytotoxic agent for tumor therapy. In this study, we used murine colorectal tumor cell line CT26 to investigate the possible Cyt D's anticancer activities. Our results showed that Cyt D can induce CT26 cell apoptosis and proliferation *in vitro* and suppressed tumor growth and angiogenesis *in vivo*, suggesting its therapeutic values in cancer treatment.

2. Materials and methods

2.1. Cell culture

Murine colorectal carcinoma cell line CT26 (CT26) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). CT26 cells were cultured in RPMI1640 (Gibical) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. The logarithmic phase cells were used for subsequent experiments.

2.2. MTT assay

For MTT assay, CT26 cells in logarithmic growth were trypsinized and harvested, and then the cells were seeded onto a 96-well plate. After 24 h, fresh RPMI 1640 medium containing different concentrations of Cyt D (0.75, 1.25, 2.5, 5.0, 7.5, 10 and 15 μg/mL) was added at 100 μL per well respectively and each concentration has 6 replicate wells. After incubation for different time intervals, 10 μL of MTT (5 mg/mL) was added to each well and the cells were further incubated at 37 °C for 4 hours. Then the supernatant was removed and 100 μL DMSO was added into each well. In the end, the absorbance (A value) at wavelength of 490 nm was measured with a microplate reader (Bio-Tek EXL808).

2.3. Detection of cell apoptosis *in vitro*

A TUNEL-based apoptosis detection was performed using TiterTACS In Situ Detection Kit (Trevigen) as described in previous study[14]. In brief, CT26 cells were seeded in 96-well plates and incubated for 16 h in the presence or absence of tested drugs at a concentration of the optimum dose decided from the above MTT results. Thereafter, cells were fixed and nick-end labeled as recommended by the manufacturer's protocol. The absorbance at 450 nm (A450 nm), corresponding to the amount of nick-ends, was normalized to the amount of cells, as evaluated from crystal violet staining (A540 nm). For purpose of clearly representation, all A450 nm/A540 nm signals were normalized to the signal obtained from unlabeled sample

(cells treated with DMSO and without nick-end labeling) as negative control. For positive control, the nuclease-treated cells were treated with TACS Nuclease after fixation, and then nick-end labeled.

2.4. Establishment of tumor models

Female BALB/c mice at 6 to 8 weeks of age were used for establishment of tumor models. To established tumor model, BALB/c mice were injected s.c. with 2×10⁶ corresponding tumor cells in the dorsal area. The animal protocols in this study were approved by the College's Animal Care and Use Committee.

2.5. Determination of optimum effective dose

CT26-bearing BALB/c mice were randomly divided into six groups ($n = 5$ for each group). Treatment started when tumor volume was about 90 mm³. These mice were injected i.v. with various does of Cyt D (12.5, 25, 50 and 100 mg/kg in 200 μL DMSO) and DMSO (200 μL), respectively. All these reagents were given every 3 days for 15 days. The mice were sacrificed on day 18, and tumor tissues were excised and weighed. The optimum effective dose of Cyt D for further experiments was decided according to the tumor weights.

2.6. *In vivo* observations of antitumor activities

Tumor-bearing mice injected with CT26 cells were divided into two groups ($n = 10$ for each group) and i.v. injected with Cyt D (50 mg/kg in 100 DMSO) and DMSO (100 μL, as negative control) very 3 days for 21 days, respectively. The tumor volumes and survival time were observed. Tumor volumes were calculated according to the formula: $V = 0.52a \times b^2$, where a is the largest superficial diameter and b is the smallest superficial diameter. The mice were sacrificed when they became moribund and the sacrificed date was then recorded to calculate the survival time. In addition, tumor tissues were also excised, fixed in 10% formalin and frozen in -80 for detections of microvessel density.

2.7. Detection of microvessel density in tumor tissues

For microvessel density (MVD) analysis in tumor tissues, frozen sections were fixed in acetone, incubated, and stained with an antibody reactive to CD31 as we previously done[15]. The sections were then stained with labeled streptavidin biotin reagents (Dako LSAB kit, peroxidase; Dako). Vessel density was determined by counting the number of microvessels per high-power field (hpf) in the sections.

2.8. Alginate encapsulation assay

Alginate encapsulated tumor cell assays were performed as previously described[16]. Briefly, CT26 cells were

resuspended in a 1.5% solution of sodium alginate and added dropwise into a swirling 37 °C solution of 250 mM calcium chloride. Alginate beads were formed containing approximately 1×10^5 tumor cells per bead. Experiment mice were then anesthetized, and four beads were implanted subcutaneously into an incision made on the dorsal side. Incisions were closed with surgical clamps. After 14 days, mice were injected intravenously with 100 μ L of a 100 mg/kg FITC–dextran solution (Sigma). Beads were surgically removed and FITC–dextran quantified against a standard curve of FITC–dextran.

2.9. Statistical analysis

An unpaired Student's *t*-test was used to compare the statistical differences in the experimental values between two samples. Survival curves were constructed according to the Kaplan–Meier method and statistical significance was determined by the log–rank test. A *P* values < 0.05 was considered statistically significant. Error bars represent SEM unless otherwise indicated.

3. Results

3.1. Inhibition of cell proliferation by Cyt D

MTT assay was used to determine the cell proliferation. The results showed that Cyt D treatment caused significantly inhibition of cell proliferation *in vitro*. Compared with the CT26 cells treated with the control DMSO, the CT26 cells treated with Cyt D showed significant suppression of cell proliferation and the suppression was in a pattern of dose and time dependent manner (Figure 1). The percentage inhibition of CT26 cell proliferation by 0.75 and 10 μ g/mL Cyt D was (12.3 ± 2.3) % and (73.9 ± 7.1) %, respectively (Figure 1A). Moreover, when CT26 cells were treated by 10 μ g/mL Cyt D, the percentage inhibition was (41.2 ± 4.2) % for 24 hours and (68.3 ± 6.9) % for 96 hours (Figure 1B). When compared with the DMSO–treated CT26 cells, the values from the Cyt D–treated CT26 cells in every dose (Figure 1A) and at every time point (Figure 1B) showed significant difference (*P* < 0.05 or less).

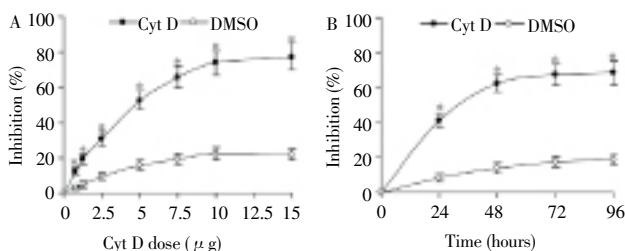


Figure 1. Inhibition of cell proliferation by Cyt D.

CT26 cells were cultured in various dose of Cyt D (0.24–15 μ g/mL). Cell proliferation was detected by Proliferation ELISA kit. Data are expressed as means \pm SEM, **P* < 0.01 or less, compared to the various dose or DMSO–treated CT26 cells.

3.2. Induction of cell apoptosis *in vitro* by Cyt D

A TUNEL–based assay that detects the amount of nick–ends generated as a result of DNA fragmentation during apoptosis was performed in a commercial Kit and the results were relatively compared to the negative control (as 1) (Figure 2). The amount of nick–ends in the CT26 cells treated with DMSO was low (1.28 ± 0.18), with a similar level as the unlabeled negative cells. In contrast, in CT26 cells treated with Cyt D the absorbance signals were significantly increased and almost reached the level in the nuclease–treated positive cells (5.28 ± 0.46 versus 5.91 ± 0.63), suggesting Cyt D is a potential effective cytotoxic agent for cancer therapy.

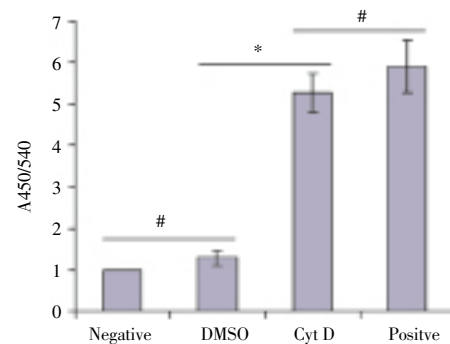


Figure 2. Induction of cell apoptosis *in vitro* by Cyt D.

CT26 cells were treated for 16 h with Cyt D or DMSO. Thereafter, cells were fixed and nick–end labeled. The amount of nick–ends (A450 nm) was divided by the amount of cells as evaluated by crystal violet staining (A540 nm). Graph shows the results of a representative experiment run in triplicate and expressed as means \pm SEM, **P* < 0.001, # > 0.05.

3.3. The optimum effective dose of Cyt D

In order to choose an optimum effective dose of Cyt D for use in the *in vivo*, CT26–bearing BABL/c mice were treated with Cyt D at different dose. Our results demonstrated that 12.5 to 100 mg/kg Cyt D showed in a pattern of dose–dependent (Figure 3). The mice treated with 12.5 and 25 mg/kg Cyt D showed somewhat inhibition of tumor growth. However, the mice treated with 50 and 100 mg/kg have the best effects in suppression of tumor growth (Figure 3). Thus, we selected the dose of 50 mg/kg as the optimum effective dose and used for subsequent *in vivo* experiments.

3.4. Inhibition of tumor growth by Cyt D

The antitumor activities of Cyt D were observed *in vivo* in CT26 tumor model. CT26–bearing BABL/c mice were treated with Cyt D and DMSO, respectively, and tumor volumes (Figure 4A) and survival time (Figure 4B) were observed. Compared with the mice treated with DMSO, the mice treated with Cyt D were showed significant inhibition of tumor growth and prolong survival time (Figure 4). Both tumor

volume and survival time were found significant differences at day 16 after CT26 tumor cell injection (Figure 4, $P < 0.01$ or less).

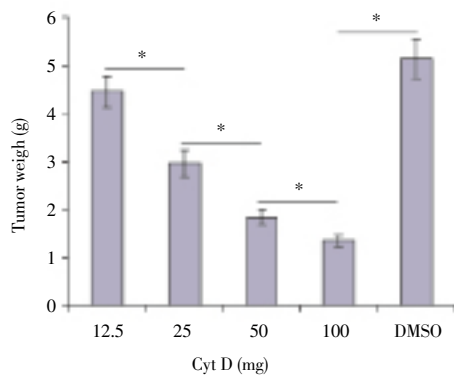


Figure 3. Decision of the optimum effective dose of Cyt D. CT26 mice were randomly divided into five groups ($n = 5$) and injected with the indicating does of Cy D or DMSO every 3 days for 15 days. The results showed the tumor weight on day 18. Data are expressed as means \pm SEM, $*P < 0.01$.

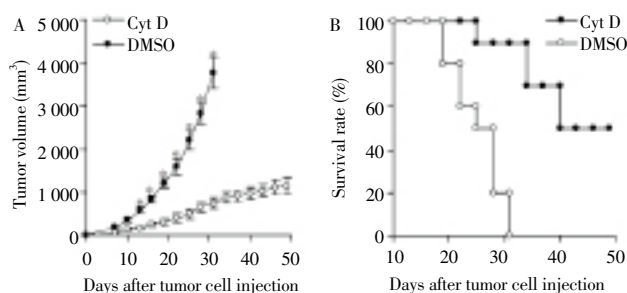


Figure 4. Inhibition of tumor growth by Cyt D.

CT26 model mice were randomly divided into two groups ($n=10$) and injected i.v. with Cyt D and DMSO, respectively. Tumor volume (A) was significantly inhibited and prolonged survival rate (B) was found in the Cyt D mice when compared with the DMSO control mice. Data are expressed as means \pm SEM, $*P < 0.05$ or less.

3.5. Inhibition of angiogenesis by Cyt D

Angiogenesis within tumor tissues was estimated through counting the number of microvessels on the sections stained with an antibody against to CD31. Compared with mice treated with DMSO, the average number of microvessels per high-power field (hpf) was significantly decreased in the mice treated with Cyt D (Figure 5A), 25.71 ± 2.94 in the Cyt D-treated mice versus 69.23 ± 4.62 in the DMSO-treated mice (Figure 5B, $P < 0.001$). In addition, the inhibition of angiogenesis in the mice treated with Cyt D was confirmed in alginate encapsulation assay. The alginate-implanted angiogenesis was quantified by measuring the uptake of FITC-dextran into the beads. The FITC-dextran uptake was also significantly decreased in the mice treated with Cyt D when compared with the mice treated with DMSO, 1.71 ± 0.23 (μ g/bead) in the Cyt D-treated mice versus $4.32 \pm$

0.48 in the DMSO-treated mice (Figure 5C, $P < 0.001$). These results indicated that the anticancer activities of Cyt D were related to the capabilities of inducing inhibition of tumor angiogenesis.

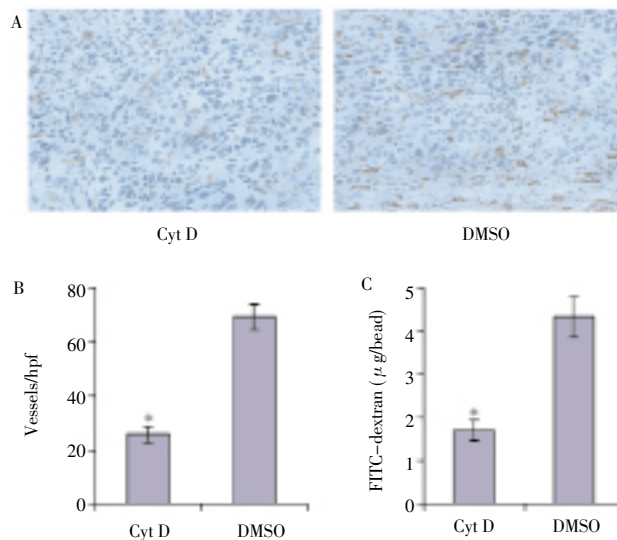


Figure 5. Inhibition of angiogenesis by Cyt D.

Frozen sections of tumor tissues were tested by immunohistochemical analysis. The vasculatures in the tumor tissue were shown (A) and quantified (B). Alginate beads containing tumor cells were implanted into the mice, and the mice were then treated with Cyt D or DMSO. Thereafter, FITC - dextran in the beads was quantified (C). Data are expressed as means \pm SEM, $*P < 0.001$.

4. Discussion

Cytochalasins are mainly isolated from tropical symbiotic fungi in tropical plants. When they were first isolated, scientists were considered them as possible cytotoxic agents and tried to use in cancer therapy. Several lines of evidence have demonstrated that cytochalasins can act on cell and tissue morphology and function *in vitro* among normal and cancer cells[17-22]. Cytochalasins can affect many cell functions, including adherence, cell motility, drug efflux, secretion, indicating that the cytochalasins might induce significant responses in experimental cancer chemotherapy model systems either as individual agents or, more likely, as agents amplifying responses induced by known antitumor drugs[14]. Up to now, many study results have indicated that cytochalasin D (Cyt D) is the most specific cytochalasins acting on the microfilaments. Thus, it is conceivable that Cyt D may potentially be a cytotoxic agent for cancer therapy. In this study, we used murine colorectal tumor cell line CT26 to investigate the possible Cyt D's anticancer activities *in vitro* and *in vivo*. Results from cell proliferation assay by MTT method showed that Cyt D inhibited CT26 cell proliferation in a dose and time dependent pattern, and the climax inhibition ratio reached almost at 80 percent.

A TUNEL-based assay to detect the CT26 cell apoptosis *in vitro* indicated that Cyt D had strong capabilities of inducing CT26 cell apoptosis, Cyt D (7.5 μ g/mL) induced CT26 tumor cell apoptosis almost reaching the level induced by positive control nuclease. As we know, inhibition of tumor cell proliferation and induction of tumor cell apoptosis are two major mechanisms by which cytotoxic agents act on cancer cells. Chemotherapy, radiation therapy, and immunotherapy all rely heavily on inhibition of proliferation and apoptosis to kill cancer cells^[23-25]. Thus, our *in vitro* results in the current study suggest that Cyt D is an excellent cytotoxic agent and has the potential prospects for using in cancer therapy.

Although *in vitro* results in our current study suggest Cyt D is a potential cytotoxic agent to CT26 tumor cells, it still keep uncertain whether Cyt D can suppress tumor growth *in vivo*. To answer this question, we established CT26 tumor model in BALB/c mice and used Cyt D as a therapeutic agent to investigate the Cyt D's anticancer activities. We found that Cyt D 50 mg/kg could significantly inhibit tumor growth, tumor weigh and tumor volume in the tumor model mice treated with Cyt D showed significantly decreased when in comparison with the mice treated with the control DMSO. The survival time in the mice treated with Cyt D was also significantly longer than those mice treated with control DMSO. In addition, our results also showed that Cyt D could suppress tumor angiogenesis in CT26 tumor model. Results from direct observation of tissue used immunohistochemistry method and alginate capsulated tumor cells to quantify the uptake of the FITC-dextran showed significant decreased blood vessels in the mice treated with Cyt D, when compared with the control mice treated with DMSO. Taken together, our results indicate that Cyt D may be a potential cytotoxic agent for treatment of colorectal tumor.

As we know, the growth and persistence of solid tumors and their metastases depend majorly on angiogenesis^[26, 27]. Therefore, anti-angiogenic therapy, which targets genetically stable endothelial cells, is also an optimal alternative for the treatment of tumors. Combination of these two strategies for tumor therapies has indicated a promising advantage in many previous preclinical studies^[28-30]. The results in our current study showed that Cyt D could induce inhibition of CT26 tumor angiogenesis. Although our results in this study do not unveil the possible mechanism by which Cyt D acts on the tumor angiogenesis, many previous studies indicated that almost all cytotoxic agents clinically used for cancer therapy have the capabilities of inhibition of tumor angiogenesis even in small dose. Tumor angiogenesis is related to endothelial cells, which are active cells as cancer cells, cytotoxic agents thus can kill the endothelial cells in the same mechanisms. Our *in vitro* results indicated that Cyt D could inhibit CT26 cell proliferation and induce cell apoptosis; we thus consider that both inhibition of cell

proliferation and promotion of cell apoptosis by Cyt D are involved in the processes of inhibiting tumor angiogenesis *in vivo*. However, the exact molecular mechanisms behind these phenomena induced by Cyt D remain unknown and need to be further investigated in the future.

Cyt D is a fungal metabolite that is capable of permeating cell membranes, binding to actin and altering its polymerization. At present, Cyt D is reported to be the most specific for the actin cytoskeleton among cytochalasins^[13]. As we know, the Cyt D's targets, microfilaments, are ubiquitous in eukaryotic cells, if Cyt D is considered to be used as a cytotoxic agent, it will be possible to induce extensive side effects. In this study, although we found Cyt D is possible an effective cytotoxic agent for treatment of CT26 tumor, we did not perform experiments to investigate its side effects. Thus, detailed and systematic studies are need to be carried out to investigate the possible side effects when consider using Cyt D as an anticancer agent in the further.

In summary, we used murine colorectal tumor cell line CT26 to investigate the possible anticancer activities of Cyt D *in vitro* and *in vivo*. Our study results suggested that the Cyt D could effectively induce inhibition of CT26 tumor cell proliferation and cell apoptosis *in vitro*, and caused tumor suppression of CT26 tumor growth and angiogenesis *in vivo*. Thus, Cyt D may be potentially used as a cytotoxic agent for cancer therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

Role of the funding source

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