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• COLORECTAL CANCER •

Effects of TNP-470 on proliferation and apoptosis in human colon cancer xenografts in nude mice

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Abstract

AIM: To study the effect of TNP-470 on cell growth, proliferation and apoptosis in human colon cancer xenografts in nude mice.

METHODS: Human colon cancer xenografts were transplanted into 20 nude mice. Mice were randomly divided into two groups. TNP-470 treated group received TNP-470 (30 mg/kg, s.c) every other day and the control group received a sham injection of same volume saline solution. They were sacrificed after 4 weeks and their tumors were processed for histological examination. The expression of proliferating cell nuclear antigen (PCNA) in tumors was detected using immunohistochemical method with image analysis, and apoptosis in tumor cells was measured by TdT-mediated biotinyated-dUTP nick end labeling (TUNEL) staining.

RESULTS: Comparing with controls, tumor growth was significantly inhibited in TNP-470 treated group, the inhibitory rate being 54.4 %. Expression of PCNA in tumors of TNP-470 treated group (PI 54.32±11.47) was significantly lower than that of control group (PI 88.54±12.36), $P<0.01$. Apoptosis index (AI) of TNP-470 treated group (18.95±1.71) was significantly higher than that of control group(7.26±1.44), $P<0.001$, typical morphological change of apoptosis in tumor cells was observed in TNP-470 treated group.

CONCLUSION: Besides the anti-angiogenic effects, TNP-470 can inhibit tumor growth by inhibiting the proliferation and inducing apoptosis of tumor cells.

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INTRODUCTION

The worldwide incidence of colorectal cancer is estimated at 945 000 patients per year. Many of these patients present with metastases. The past decade has seen several extensive investigations into advanced colorectal cancer. However, Most patients have limited improvement in long-term prognosis[1]. Therefore, new therapeutic methods are needed to treat and improve the survival rate in colorectal cancer patients.

Tumors are always dependent on the development of an adequate blood supply through angiogenesis for growth at both primary and secondary sites, and conlon cancer would not be an exception[2-9]. The concept of anti-angiogenesis therapy was first proposed by Folkman[10,11]. The implications of angiogenesis for tumor biology and therapy were investigated, and some anti-angiogenesis agents have been developed[12-14]. TNP-470 is a potent angiogenesis inhibitor and an analogue of fimagillin, which is a natural antibiotic secreted by *Aspergillus fumigatus fresenius*[15,16]. This agent shows a marked inhibitory effect on tumor growth and metastasis *in vivo*[17-19]. Its target is not only the endothelial cells but also the cancer cells of the host, and the tumor is affected directly[20-22]. In this study, we examined the inhibitory effect of TNP-470 on tumor growth of human colon cancer xenografts in nude mice. The expression of proliferating cell nuclear antigen (PCNA) in tumors was examined by immunohistochemical method and apoptotic cancer cells were measured by TUNEL assay.

MATERIALS AND METHODS

Drug and reagents

TNP-470 was a generous gift from Takeda Chemical Industries (Osaka, Japan). Its structure and characteristics have been reported. TNP-470 was suspended in a vehicle composed of 0.5 % ethanol plus 5 % gum Arabic in saline. RPMI 1640 and heat-inactivated fetal calf serum (FCS) were purchased from Gibco(Grand Island, NY).

Cell line

Human colon adenocarcinoma cell line Lovo was kindly provided by the Department of Pathology, Cancer Center, First Military Medical University (FIMMU). Cells were cultured in RPMI 1640 supplemented with 10 % FCS, and were maintained at 37 ℃ in 5 % CO_2. A single cell suspension of approximately 5×10^6 cells in 0.5 mL of culture medium was inoculated subcutaneously in two Balb/c nude mice to make source tumors. These source tumors were excised when they grew to approximately 1 cm^3, and then 2 to 3 mm^3 of minced tumor tissue was implanted subcutaneously into the left axial region of the cervix of each balb/c nude mouse on day 0.

Animals

Female Balb/c nude mice were obtained from the Experimental Animal Center, FIMMU, and reared under specific pathogene-free conditions. Four-week-old mice weighing 17-22 g were used in the experiments. On day 1 after implantation, tumor-bearing nude mice were randomly divided into a control group (n=10) and a TNP-470 treated group (n=10). In the TNP-470 treated group, TNP-470 of 30 mg/kg was injected subcutaneously every other day from day 1 until sacrifice. In the control group, mice received a sham injection of the same volume of saline. The average tumor volumes and animal

weights in the treatment and control groups were almost equal at the beginning of treatment.

Tumor growth and animal weights

The tumor dimensions were measured every 3 days with a dial caliper. The tumor volumes were calculated with the formula width 2 length 0.52. Mice were weighed every 3 days. The animals were painlessly killed on day 30. All animals were weighed before autopsy, at which time, the tumor weight and volume were obtained. The tumor volume was also expressed by the rate of the mean tumor volume in that treated animals to the mean tumor volume in that of control animals (T/S rate). Tumor tissues were resected and fixed intact with 10 % formalin solution, and then cut into four-micrometer-thick sections for PCNA immunohistochemical assay and TUNEL staining.

Immunohistochemical detection of PCNA

Four-micrometer-thick sections were incubated with mouse monoclonal antibodies against PCNA (Gibco, Grand Island, NY) as the primary antibody, followed by biotinylated anti-mouse immunoglobulin and avidin-biotin complex. The PCNA indices were calculated with image analysis system as the percent rate of positively stain Lovo cell nuclei to the total of Lovo nuclei. A minimum of 500 cells was counted in triplicate[23].

TUNEL staining detection of apoptotic Lovo cells

We stained tumor tissue sections with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) to identify apoptotic cells[24]. Briefly, the sections (after being dewaxed in xylene and rehydrated in ethanol) were incubated with 20 μg/L proteinase K at room temperature for 15 minutes. After quenching of endogenous peroxidase, sections were rinsed in TdT buffer (30 mM Tris, 140 mM sodium cacodylate, 1 mM cobalt chloride), pH 7.2, and incubated with TdT (Pharmacia Biotech, Piscataway, NJ, USA) 1:50 and biotinylated dUTP (Gibco, Grand Island, NY, USA) 1:50 in TdT buffer for 60 min at room temperature. Labeled nuclei were detected with DAB in PBS and counterstained with methyl green for 10 minutes and 5 minutes, respectively. The slides were then mounted and examined under light microscope. Negative controls were tissues processed with omission of the TdT reaction step. Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively. The apoptotic cells and bodies were counted in 10 high-power fields, and this figure was divided by the number of cells in the high-power fields with image analysis system.

Statistical analysis

Data were expressed as mean ± standard deviation. Comparison between two groups was made by the independent samples *t* test. $P<0.05$ was considered statistically significant. All statistics were carried out using SPSS10.0 statistics software.

RESULTS

Antitumor effects

Before treatment, the mean tumor volumes in the two groups were approximately the same. The mean tumor volume and tumor weight of two groups at the end of the experiment are shown in Table 1. Tumor growth curve is illustrated in Figure 1. Significant inhibitory efficacy was obtained in TNP-470 treated group.

Proliferation and apoptosis of lovo cells

In PCNA immunohistochemical analysis and TUNEL staining, the number and extent of apoptotic cells were considerably increased and PCNA positive cells were decreased in the TNP-470 treated group compared with those on the control group. The proliferation index and apoptosis rate of Lovo cells in two groups are shown in Table 2.

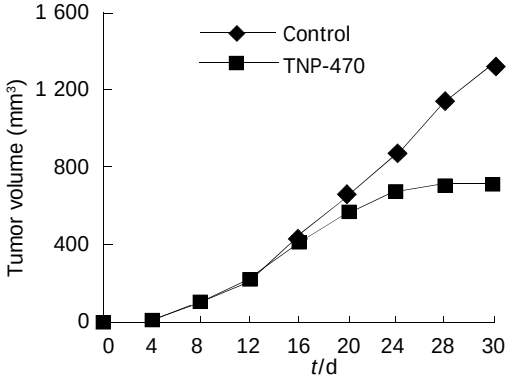

Figure 1 Tunor growth curve.

Table 1 Tumor weight and tumor volume in two groups ($\bar{x}\pm s$)

Group	*n*	Tumor weight (g)	Tumor volume(mm³)	T/C(%)
Control	10	1.423±0.169	1345.24±38.46	100
TNP-470	10	0.830±0.123[a]	731.66±40.42[b]	54.4

[a]$P<0.05$; [b]$P<0.01$, *vs* control.

Table 2 Proliferation index (PI) and apoptosis index (AI) in two groups ($x\pm s$)

Group	*n*	PI (%)	AI(%)
Control	10	88.54±12.36	7.26±1.44
TNP-470	10	54.32±11.47[a]	18.95±1.71[b]

[a]$P<0.01$; [b] $P<0.001$, *vs* control.

DISCUSSION

TNP-470 has been reported to be highly effective against a wide variety of tumors and metastases. This agent mainly exerts its anti-tumor effect by preventing tumor neovascularization[25-27]. Angiogenesis is essential for the growth of solid tumors at primary and at secondary sites[28]. It is thought that the new blood vessels in tumor are highly permeable and provide a route for cancer cells to enter the circulation[29]. Therefore, anti-angiogenesis agents may have the potential to be clinically useful for the prevention of cancer progression.

TNP-470 is well described to antiproliferate the activity against endothelial cells and anti-angiogenic properties *in vivo*. In the recent study, TNP-470 was found to suppress DNA synthesis and cause an increased proportion of cells to go into the G0/G1 phase[30-32]. A molecular target recently identified that TNP-470 is the mammalian protein MetAP-2. TNP-470 covalently attaching MetAP-2 in endothelial cells may prevent the myristylation of signaling components specific to cell cycle regulation of these cells, and inhibited the proliferation of endothelial cells[33-35]. Other studies[36,37] described that cell cycle inhibition by TNP-470 is mediated by p53 and $p21^{WAF/CIP}$.

TNP-470 has been reported to inhibit various tumor cell proliferation at concentrations much higher than those required to inhibit endothelial cells, yet the exact mechanisms underlying these effects are unclear. In contrast, there are reports that TNP-470 has no significant effect on primary tumors transplanted s. c[38]. These differences may arise because of organ-site-dependent differences in tumorigenesis and on cancer-cell properties. In addition, sensitivity to TNP-470 varied from tumors possibly due to the tumors' biological malignancy, the growth activity,

the metastasis rate, the adhesive activity to extracellular matrix and the production of VEGF[10, 39, 40].

The antitumor activity of TNP-470 on a variety of tumors was evaluated *in vitro* and *in vivo*, but not in human colon cancer Lovo cells. In the present study, we found that TNP-470 inhibited the growth of human colon cancer cell line Lovo *in vivo* with an inhibitory rate (T/C) of 45.6 % and no weight loss was observed. Another important discovery in the present study was that TNP-470 could inhibit the expression of PCNA in tumors and induce apoptosis of cancer cells *in vivo*. But mechanisms of effect are not clear. Further studies *in vitro* will be necessary.

In conclusion, the present *in vivo* results demonstrated that treatment with TNP-470 could be efficient to inhibit the growth of colon cancer. Further investigation on the mechanism of the apoptosis-inducing effect of TNP-470 will help understand the mechanisms of tumor growth and metastasis.

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