Original

Anti Tumor Effects of Thalidomide Against Liver Metastasis in a Murine Model of Colon Cancer

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Background: Thalidomide was introduced in the 1950s as a nontoxic sedative, but was removed from the market because of its teratogenicity. Recent studies have demonstrated that the most likely etiology of limb defects produced by fetal exposure to thalidomide is the inhibition of angiogenesis in the developing limb bud. Also many studies have shown that thalidomide inhibits tumor growth in several malignancies by the inhibition of VEGF. **Methods:** We determined whether the systemic administration of thalidomide inhibits colon cancer liver metastasis in mice. We also evaluated the optimal schedule of this treatment against murine colon cancer liver metastasis. Murine colon cancer CT-26 cells were implanted into the spleens of BALB/c mice. 7 days after tumor implantation, the mice received an intra-peritoneal injection of thalidomide (0, 30 mg/kg) daily or every other day (3 times per week). After the treatments, all mice were sacrificed. The numbers of liver metastases were counted and the expression of VEGF and the micro vessel density were analyzed by immunohistochemistry against liver metastases. **Results:** 4 out of 10 mice which received a daily administration of thalidomide (30 mg/kg) died. But in this group, we found a significant reduction in the number of liver metastases compared with the control group (0 mg/kg). All mice which received thalidomide every other day survived. In this group, there was also a significant reduction in the number of liver metastases compared with the control. Immunohistochemical analysis revealed lower expressions of VEGF and CD31 in the liver metastases of mice which received thalidomide every other day compared with the control. **Conclusion:** The systemic administration of thalidomide inhibits liver metastasis of colon cancer in mice by the downregulation of VEGF and angiogenesis. The every other day administration of thalidomide was the optimal schedule in this model.

Keywords: Thalidomide, VEGF, Liver metastasis, tumor vasculature
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Introduction

Colorectal cancer is the fourth most common malignancy and the second leading cause of cancer death in the United States. Despite the considerable energy spent on cancer research and the introduction of new therapies, the prognosis for colorectal cancer is still poor, with a mean overall 5 year survival rate of around 50 %[1)]. The major cause of death from colorectal cancer is liver metastasis resistant to conventional therapy[2)]. Metastasis depends on the induction of an adequate and new blood supply, i.e., angiogenesis[3)]. Vascular endothelial growth factor (VEGF) is one stimulator of angiogenesis and its expression correlates with tumor vasculature and colon cancer liver metastasis[4)].

Thalidomide was introduced in the 1950s as a nontoxic sedative, but was removed from the market because of its teratogenicity. Recent studies have demonstrated that the most likely etiology of limb defects produced by fetal exposure to thalidomide is the inhibition of angiogenesis in the developing limb bud[5)]. Thalidomide has been shown to inhibit VEGF and basic fibroblast growth factor (bFGF)-induced angiogenesis[6)]. This anti-tumor effect of thalidomide has also been reported in several clinical trials[7-10)]. In colorectal cancer, combination therapy with paclitaxel and thalidomide

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Abbreviations: FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; EMEM, Eagle's minimum essential medium; VEGF, vascular endothelial growth factor

inhibits the angiogenesis and growth of human colon cancer xenografts in mice[11]. Good response rates were also demonstrated in a combination therapy with irinotecan and thalidomide against colorectal cancer in a phase II trial[12]. However we have not yet elucidated the optimal schedule of treatment against colorectal cancer for thalidomide.

In the present study, we investigated that the efficacy of intra peritoneal injections of thalidomide inhibited liver metastasis in syngeneic mice.

Materials and methods

Thalidomide. Thalidomide was obtained from Tocris Cookson Inc. Its chemical structure has been published previously[13-16]. Dimethyl sulfoxide (DMSO) soluble thalidomide to 25 mM.

Reagents. Eagle's minimum essential medium (EMEM), Ca^{2+}-and Mg^{2+}-free Hanks' balanced solution (HBSS), and fetal bovine serum (FBS) were purchased from M.A.Bioproducts (Walkersville,MD).

Cells and cell culture. CT-26 murine colon carcinoma cells syngeneic to BALB/c mice were grown as monolayers in MEM supplemented with 5 % FBS, vitamins, sodium pyruvate, L-glutamine, and non-essential amino acids. All cultures were free of mycoplasma, the reovirus, type3 pneumonia virus of mice, K virus, ectomelia virus, and lactate dehydrogenase virus. CT-26 cells produce a high expression of murine VEGF protein and this expression is associated with colon cancer liver metastasis[17].

Experimental Liver metastasis. Specific pathogen-free male BALB/c mice were purchased from Saitama Experimental Animals Supply Corporation. Animals were maintained according to institutional guidelines for facilities approved by the Japanese Association of Laboratory Animal Care. The mice were used according to institutional guidelines when they were 10 weeks old.

CT-26 colon cancer cells in the exponential growth phase were harvested by a brief exposure to a 0.25 % trypsin −0.1 % EDTA solution. The cell suspensions were pipetted to produce single-cell suspensions, washed, and resuspended in HBSS. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions with a viability of greater than 90 % were used. Mice were anesthetized by intraperitoneal injections of Nembutal (30 mg/kg) and placed in the supine position. A midline abdominal incision was made and the spleen was exteriorized. The incision was closed in one layer with wound clips[18]. Tumor cells (5×10^3/mouse) were injected into the spleen, and at the end of the study, the mice were sacrificed. The livers were removed and placed in Bouin's solution. The number of experimental liver metastases was determined with the aid of a dissecting microscope, and the number of liver lesions smaller than 1mm in diameter was determined in representative sections stained with H and E using a light microscope.

Systemic therapy with thalidomide. On day 7 after the inoculation, the mice received an intra-peritoneal injection of thalidomide (0, 30 mg/kg) daily or every other daily (3 times per week). On day 21 after the inoculation, all mice were sacrificed. The number of liver metastases was counted and the expression of VEGF and the micro vessel density were analyzed by immunohistochemistry against liver metastases.

Histology and immunohistochemstry. Livers that contained colon cancer metastases were divided into fragments and placed in either 10 % neutral formalin (vol/vol) or OCT compound (Miles laboratories, Elkhart, IN) to be snap-frozen in liquid nitrogen. For histological study, consecutive 5-μm sections were stained with H&E. For immunohistochemical analysis, frozen sections (10 μm) were fixed with cold acetone. Tissue sections (5 μm) of formalin-fixed, paraffin-embedded specimens were treated in xylene, rehydrated in a graded alcohol series, and transferred to PBS for 12 min. Non-specific reactions were blocked by incubating the section with a solution containing 5 % normal serum and 1 % normal goat serum for 20 min at room temperature. Excess blocking solution was drained, and the slides were incubated overnight at 4℃ with antibodies to CD31 (1:400 dilution; Pharmingen, San Diego, CA), and VEGF (1:20 dilution; Oncogene, Cambridge, MA). They were then rinsed three times with PBS and incubated with peroxidase-conjugated secondary antibody for 60 min at room temperature, followed by rinsing with PBS and incubation with diaminobenzamine (Research Genetics, Huntsville, AL) for 5 min. The sections were counter-stained with Gill's hematoxylin. A positive reaction in this assay stained reddish brown due to a precipitate in the cytoplasm. The intensity of staining was quantified in three different areas of each sample by an image analyzer using the Optimas software program (Bioscan, Edmonds, WA) to yield an average measurement[19]. Endothelial cells ($CD31^+$) in ten 'hot spots'[20] of the liver tumors were counted under $\times$100 magnification microscopic fields ($\times$10 objective and $\times$10 ocular, 0.14mm^2 per field) and expressed as the number per mm^2 [21]. Based on criteria for microvessel density described by Weidner *et al*[22],

it was not necessary to observe the vessel lumen to classify the structure as a vessel. We designed negative control for immunohistochemical analysis by without secondary antibodies in these studies.

Statistical Analysis. Significant differences in the data were analyzed by the Mann-Whitney U test. P-values <0.05 were considered significant.

Results

CT-26 colon cancer liver metastasis with the systemic administration of thalidomide. We examined whether the systemic administration of thalidomide inhibits the number of experimental liver metastases. Viable 5×10^3 CT-26 cells were implanted into the spleens of mice. 7 days after tumor implantation, mice received an intra-peritoneal injection of thalidomide (0, 30 mg/kg) daily or every other day (3 times per week). The number of liver metastases was counted. 4 out of 10 mice which received a daily administration of thalidomide (30 mg/kg) died in this study. But in this group, we found a significant inhibition of the number of liver metastases compared with the control group (0 mg/kg) (Table 1).

All mice which received thalidomide every other day survived. In this group, there was a significant inhibition of the number of liver metastases compared with the control group (Table 1).

Microvessel density and the expression of VEGF in hepatic metastases with thalidomide. To determine whether thalidomide inhibits the tumor vasculatures of liver metastases, we examined microvessel density and the expression of murine VEGF by immunohistochemical analysis using antibodies against the endothelial cell marker CD31 and murine VEGF. Liver metastases exposed to a daily administration of thalidomide were significantly inhibited with regard to the formation of new blood vessels (Fig. 1). Also the average microvessel density of lesions which received thalidomide every other day was inhibited (225 ± 80 vessels/mm^2 compared with 327 ± 70 vessels/mm^2 in the control tumors) (mean$\pm$SEM) (Fig. 1, 2).

Table 1. Murine colon cancer liver metastasis with thalidomide

Treatment	Liver metastasis	
	Incidence	Meta. No Median (range)
Thalidomide (every day)		
0 mg/kg	10/10	5 (1-37)
30 mg/kg	5/6	4 (0-4)*
Thalidomide (every other day)		
0 mg/kg	9/10	13 (0-50)
30 mg/kg	8/10	4 (0-31)*

CT26 cells were inoculated into the spleens of mice. On day 7 after the inoculation, the mice received an intra-peritoneal injection of thalidomide (0, 30 mg/kg) daily or every other daily (3 times per week). The number of liver metastases was counted. *4 out of 10 mice which received a daily administration of thalidomide (30 mg/kg) died on day 4 after treatment. We stopped this experiment on day 16. All mice which received thalidomide every other day survived in this study. All mice were sacrificed on day 21.* **P<0.05* compared with the control (0 mg/kg).

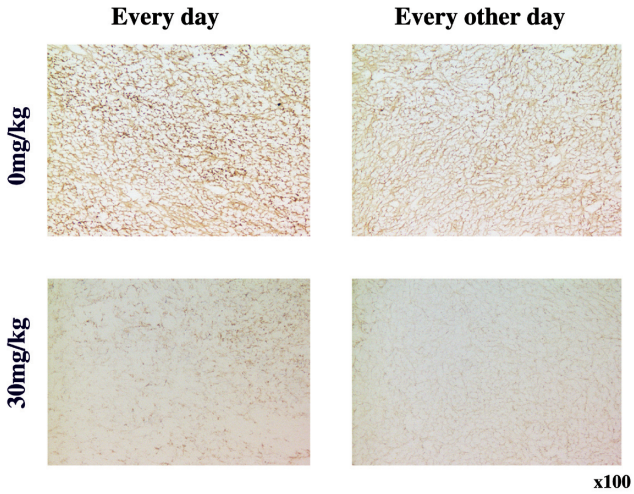

Fig. 1. Tumor vasculature of CT26 liver metastasis with thalidomide. CT26 murine colon cancer cells were injected into the spleens of mice on day 0. On day 7, the mice received a systemic administration of thalidomide (0, 30 mg/kg) daily or every other day (3 times per week). After treatment, all mice were sacrificed and the hepatic metastases were processed for the immunohistochemistry against the CD31 antibody. Tumor vasculature reductions were observed in liver tumors with thalidomide.

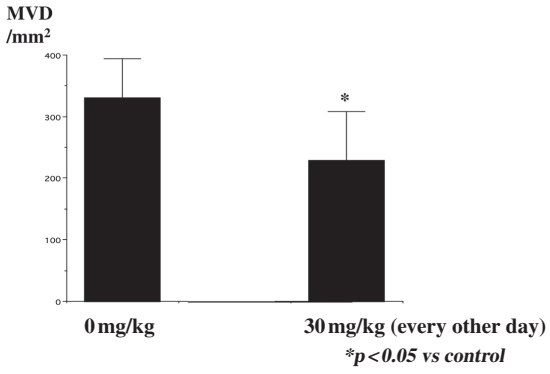

Fig. 2. MVD of hepatic metastasis with thalidomide. Endothelial cells (CD-31$^+$) in liver tumors were counted under x100 magnification microscopic fields and expressed as the number of cells per mm^2. The average microvessel density (MVD) of the lesions which received thalidomide every other day was inhibited significantly ($p<0.05$ vs control).

Also, the inhibition of VEGF protein expression correlated with a reduction in the tumor vasculature of hepatic lesions with the systemic administration of thalidomide (Fig. 3). In the normal hepatic areas, we found a lower expression of VEGF and CD31 compared with liver metastases.

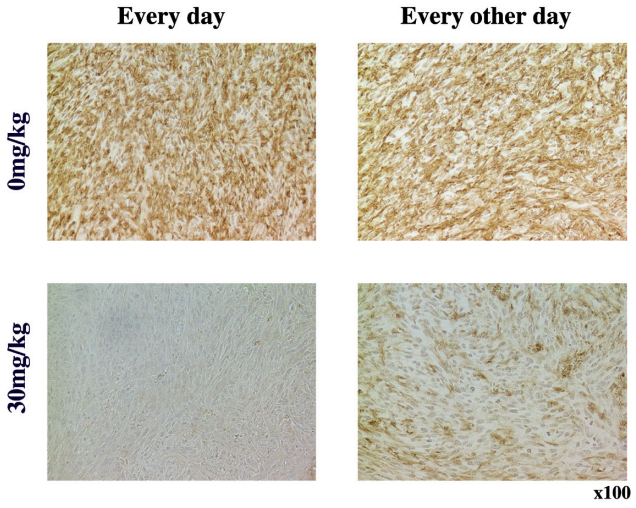

Fig. 3. VEGF expression of CT26 liver metastasis with thalidomide. CT26 cells were injected into the spleens of mice on day 0. On day 7, the mice received a systemic administration of thalidomide (0, 30 mg/kg) daily or every other day. After treatment, all mice were sacrificed and the hepatic metastases were processed for the immunohistochemistry. A lower expression of VEGF was seen in the lesions with thalidomide.

Discussion

Our results demonstrated that the systemic administration of thalidomide to syngeneic mice with liver metastasis of murine colon cancer inhibits angiogenesis associated with the inhibition of VEGF expression. Altering the time schedule of administration influenced the therapeutic outcome.

The progressive growth of primary neoplasms and metastases depends on angiogenesis[3, 23, 24], and the extent of angiogenesis is determined by the balance between proangiogenic and antiangiogenic molecules[15-17]. Angiogenesis induced by colon cancer is associated with VEGF, bFGF, IL-8, and MMP-9[4, 25-29]. The expression of VEGF correlates with tumor vasculature and colon cancer liver metastasis[4].

Thalidomide was first marketed as a sedative hypnotic in 1957. It was withdrawn from the market in 1961 as it was found to cause congenital defects[30]. Thalidomide has been shown to inhibit VEGF, basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8)-induced angiogenesis[5, 6, 31]. This anti-tumor effect has also been reported in several clinical trials[7-10].

Previous studies have shown that the major toxicity of thalidomide is a reversible sedative. Additional serious toxicities include peripheral neuropathy, thrombosis, and a rash[32-35]. Thyroid dysfunction has also been reported[36]. In a clinical trial of thalidomide in patients with Kaposi's sarcoma, the maximum tolerated dose (MTD) was 600 mg/day, with dose-limiting toxicity (DLT) of somnolence and a rash[37].

In this study, 4 out of 10 mice which received a daily administration of thalidomide (30 mg/kg) died on day 4 after treatment. In our preliminary studies, all mice which received a daily administration of thalidomide (300 mg/kg) died on day 4 after treatment (data not shown).We suggest that the cause of death was the suppression of breathing due to somnolence in mice. All mice which received thalidomide every other day survived in this study. In preliminary studies, we administered thalidomide (3 mg/kg) every other day. There were no significant inhibitions of liver metastasis and the expression of VEGF compared with the control (data not shown).

In our study intra peritoneal administration was employed as previously described[38]. Kotoh et al.[39] reported the antiangiogenic effect of thalidomide on human esophageal cancer in nude mice by intraperitoneal administration. Whereas same effect were not observed when mice were treated by gavage administration. In clinics, thalidomide is used by oral administration. However, single-agent thalidomide is generally well-tolerated drug that showed no antitumor activity in patients with advanced metastatic colorectal cancer[40]. The mechanisms underlying the strong effect of antiangiogenesis by intraperitoneal root but poor efficiency by oral route remain unclear, the reasons might be the bioavailability of the active form of thalidomide at tumor site.

In summary, an every other day administration of thalidomide (30 mg/kg) is optimal schedule for this model. This schedule produced the highest efficacy against colon cancer liver metastasis and its therapeutic effect was correlated with the reduction of tumor vasculature in mice. To improve the prognosis and safety of clinical trials of thalidomide against colon cancer liver metastasis, we need to develop an optimal dose and schedule in the future.

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マウス大腸癌肝転移モデルに対するサリドマイド治療

小澤　修太郎，俵　英之，小山　勇

サリドマイドは妊婦の入眠剤として開発された薬剤であり当時，胎生期，四肢の血管新生阻害を介した催奇形性が認められた．その後の研究で様々な癌腫におけるVEGF等の発現抑制を介した抗腫瘍効果が確認された．今回，我々はマウス大腸癌肝転移モデルを作成．サリドマイドの腹腔内注射を行い，肝転移に対する最適投与計画を確立したので報告する．マウス大腸癌CT-26細胞をBALB/cマウス脾臓に移植して肝転移モデルを作成．サリドマイド(0, 30 mg/kg)を連日および隔日で腹腔内注射．治療終了後，肝転移数，転移性腫瘍内のVEGF蛋白，血管新生発現を免疫組織学的染色で比較検討した．連日投与群は10匹中4匹死亡したがコントロール群に比べ肝転移数の発現抑制が見られた．30 mg/kg隔日投与群は治療中の死亡はなく，コントロール群に比べ有意に肝転移の抑制が見られた．又，転移性肝腫瘍内のVEGF蛋白および微小血管新生の発現抑制が免疫組織学的染色で確認された．サリドマイド30 mg/kgの隔日腹腔内投与はマウス大腸癌肝転移モデルに対し有効であり，この治療計画は本モデルにおける最適治療計画であった．本治療効果はVEGF蛋白発現制御を介したものであることが示唆された．

埼玉医科大学 消化器・一般外科（I）教室 〔平成17年1月25日受付〕