PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Effects of thalidomide on angiogenesis and tumor growth and metastasis of human hepatocellular carcinoma in nude mice

Zhong-Lin Zhang, Zhi-Su Liu, Quan Sun

Zhong-Lin Zhang, Zhi-Su Liu, Quan Sun, Department of General Surgery, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China
Correspondence to: Professor Zhi-Su Liu, Department of General Surgery, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China. zhangzhonglin221@sina.com
Telephone: +86-27-67813007 **Fax:** +86-27-87330795
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Abstract

AIM: To investigate the effects of thalidomide on angiogenesis, tumor growth and metastasis of hepatocellular carcinoma in nude mice.

METHODS: Twenty-four nude mice were randomly divided into therapy group and control group, 12 mice in each group. Thalidomide dissolved in 0.5% sodium carboxyl methyl cellulose (CMC) suspension was administered intraperitoneally once a day at the dose of 200 mg/kg in therapy group, and an equivalent volume of 0.5% CMC in control group. Mice were sacrificed on the 30th d, tumor size and weight and metastases in liver and lungs were measured. CD34 and VEGF mRNA in tumor tissue were detected by immunohistochemistry and semi-quantitative RT-PCR respectively and microvessel density (MVD) was counted. Serum concentrations of TNF-α and ALT and AFP were also tested.

RESULTS: MVD and VEGF mRNA in therapy group were less than those in control group (31.08±16.23 vessels/HP *vs* 80.00±26.27 vessels/HP, 0.0538±0.0165 *vs* 0.7373±0.1297, respectively, $P<0.05$). No statistical difference was observed in tumor size and weight and metastases in liver and lungs. TNF-α was significantly lower in therapy group than in control group (28.64±4.64 ng/L *vs* 42.69±6.99 ng/L, $P<0.05$). No statistical difference in ALT and AFP was observed between groups.

CONCLUSION: Thalidomide can significantly inhibit angiogenesis and metastasis of hepatocellular carcinoma. It also has inhibitory effects on circulating TNF-α.

Key words: Hepatocellular carcinoma; Thalidomide; Angiogenesis; Neoplasm metastasis

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INTRODUCTION

Malignant tumor's growth, invasion and metastasis depend on the process of angiogenesis[1,2], obliteration of the feeding vessels to a tumor could cause its shrinkage or death[3]. As a result, antiangiogenic therapy has become a hotspot in the field of tumor treatment. Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer death, and accounts for 53% of all liver cancer deaths in China[4]. Poor prognosis of hepatocellular carcinomas is mainly due to its high recurrence and metastasis. HCC also a kind of typical hypervascular malignant tumor, for which antiangiogenic therapy is particularly promising.

It has been found in a recent research that thalidomide, having been removed from medical markets for its severe side effects of teratogenesis, has antiangiogenic effects[5]. Thalidomide first entered medical care markets as a non-barbital sedative with remarkable anti-emetic effects on nausea of first-trimester morning sickness in pregnant women. Unprecedented epidemic of babies' birth defects in late 1950s and early 1960s was due to its serious potential side effects of teratogenicity, and then the drug has been prohibited and removed from markets since 1963. But research on this agent has never stopped and in 1994 D'A mato *et al*[5] firstly reported it could remarkably reduce neovascularization in rabbit corneas after stimulation by basic fibroblast growth factor (bFGF), and following studies[6-8] confirmed its antiangiogenic effects. As a result, thalidomide has been employed in the studies of solid tumor as an antiangiogenic agent in recent years. Some preclinical and clinical trials for the treatment of several types of solid tumor using thalidomide have been reported[9-11]. However, the effectiveness and mechanism of this antiangiogenic agent for the treatment of hepatocellular carcinoma have not been fully investigated. In the current study we established nude mice models bearing xenografts of human hepatocellular carcinoma with a high metastatic potential, by which we examined the effect of thalidomide on angiogenesis and tumor growth and metastasis of human hepatocellular carcinoma. Its influence on tumor necrosis factor α (TNF-α) and liver function was also investigated.

MATERIALS AND METHODS

Animals

Male athymic BALB/c nu/nu mice, 4-6 wk old, were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences and maintained under specific pathogen-free (SPF) conditions. The study protocol on mice was approved by Shanghai Medical Experimental Animal Care Commission.

Metastatic model of human HCC in nude mice

Human hepatocellular carcinoma cell line HCCLM3 was established by Liver Cancer Institute of Fudan University[12], in which a metastatic model of human hepatocellular carcinoma in nude mice was constructed via orthotopic implantation of histologically intact metastatic tumor tissue. Briefly, HCCLM3 derived 5×10^6 (0.2 mL) cells were injected subcutaneously into the nude mice. When the subcutaneous tumor reached about 1.5 cm in diameter, mice were sacrificed and small pieces of tumor tissue (approximately 1 mm^3) were implanted into the

liver of new recipient mice, which were kept in standard facilities. This animal model represents 100% spreading in liver and metastasis to lungs. Besides, alpha-fetoprotein (AFP) was excreted and hepatitis B virus was integrated into host cellular genome as previously reported[12].

Grouping and drug administration

Twenty-four nude mice were randomly divided into therapy group and control group, 12 mice in each group. Thalidomide was dissolved in 0.5% sodium carboxyl methyl cellulose (CMC) as an even suspension due to its poor solubility in water. Thalidomide (200 mg/kg·d) was intraperitoneally administered once a day in the therapy group and an equivalent volume of 0.5% CMC suspension simply in the control group. The injection started from the second day of inoculation and continued in the following consecutive 30 d. Body weight of mice was recorded once a week.

Parameters observed

On the 30th d all mice were sacrificed and 1 mL of blood sample was collected. After separated, tumors were weighed and the longest (a) and the smallest (b) diameters were measured by slide gauge under operating microscope. Tumor volume was calculated with the following formulation: $V = a \cdot b^2/2$. Liver tissues were carefully anatomized and visible metastases were counted. Paraffin blocks of 10% buffered formalin-fixed samples of lungs were prepared. Each lung sample was consecutively cut into 10 slices. Serial sections were cut at 5-μm and stained with hematoxylin and eosin to determine the presence of lung metastases. After blood samples were coagulated, centrifugation at 2 000×g for 10 min was performed and serum was obtained for the test of AFP and alanine aminotransferase (ALT) and TNF-α by radioimmunoassay and immunosorbent assay respectively. Part of each tumor tissue was embedded in paraffin block for advanced immunohistochemistry analysis of CD34 and the rest was stored at -70 ℃ for following RT-PCR study.

Immunohistochemical assessment of vascular density

Paraffin-embedded tumor tissues were sectioned (4 μm) and the slides were deparaffinized as usual and washed with tris buffered saline (TBS), and then incubated with 10% normal goat serum (Zhongshan Bio. CA). Sections were then incubated with appropriately diluted (1:10) rat-anti-mouse CD34 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 24 h at 4 ℃. Primary antibody was removed and washed with TBS, goat-anti-rat IgG peroxidase (Zhongshan Bio. CA) was then added. Finally the slices were stained as usual with haematoxylin and washed with distilled water. Quantification of blood vessels was carried out as previously described[13]. Any brown-stained endothelial cell cluster distinct from adjacent microvessels, tumor cells, or other stromal cells was considered as a single countable microvessel. The most vascularized areas of tumors were identified in a low-power field (×100), and vessels were counted in five high-power fields (×200). The data were presented as mean±SD of five high-power fields.

Semi-quantitative reverse transcription-PCR

Total RNA was extracted with Trizol reagent (Promega, USA) following the manufacturer's instructions and quantitated by absorbance analysis at 260 nm. For the reverse transcription polymerase chain reaction, RNA PCR kit (AMV) (TakaRa Bio, JP) was used. Total volume of reverse transcription reaction was 10 μL. Reaction temperature was 30 ℃ for 10 min, 42 ℃ for 20 min and 45 ℃ for 30 min. For PCR reaction the total reacting volume was 50 μL. PCR reaction was performed in GeneAmp PCR system 2400 (Perkin Elmer, USA). Primers were designed according to previous publications[14,15]. Primer sequences and PCR reaction conditions are shown in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as the internal standard. Of the PCR products 5 μL was visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide and quantitated by densitometry using the Image Master VDS system and associated software (Pharmacia, Sweden).

Enzyme-linked immunosorbent assay for TNF-α

Serum TNF-α was tested by enzyme-linked immunosorbent assay (ELISA) using TNF-α ELISA kit (Basic Medical Institute of Shanghai, Chinese Academy of Military Medical Sciences). Procedure was designed according to manufacturer's instructions, concentrations of unknown samples were determined by comparing the optical density of samples to the standard curve.

Statistical analysis

Data were analyzed for significance with unpaired *t* test and chi-square test. Statistical software SPSS 11.5 was used in the analysis. *P* value less than 5% was considered statistically significant.

RESULTS

Effects of thalidomide on growth of HCC

Lumps in stomach and skin invasion could be observed at the 5th wk when mice were sacrificed. The separated tumors are shown in Figure 1. The changes of body weight (g) and tumor weight (g) and tumor volume (cm^3) in treatment group were all smaller than those in control group (4.3000±1.9980 *vs* 5.1833±0.9827, 1.0333±0.2842 *vs* 1.1483±0.3633 and 0.9950±0.3987 *vs* 1.2806±0.3188, respectively) There was no statistical significance ($P>0.05$).

Effects of thalidomide on metastasis of HCC

Visible metastases ranging from 1 to 10 mm in diameter were observed when seven lobes of liver were carefully anatomized and the number of visible metastases was recorded. Gross pathological examination of the lungs found scattered hemorrhagic spots, which were confirmed by histopathology to be metastases (Figure 2). The metastatic rate in liver and lungs in treatment group and control group was both 100%, but the number of metastases in both liver and lungs in therapy group was significantly less than that in control group ($P<0.05$, Figure 3).

Table 1 Primer sequences and PCR reaction conditions

Gene	Primers	Products size	Annealing Temp/Time	Cycles
VEGF (all isoforms)	Upper: CCTGGTGGACATCTTCCAGGAGTACC Lower: GAAGCTCATCTCTCCTATGTGCTGGC	196 bp	58 ℃/30 s	30
G3PDH	Upper: ACCACAGTCCATGCCATCAC Lower: TCCACCACCCTGTTGCTGTA	450 bp	58 ℃/30 s	30

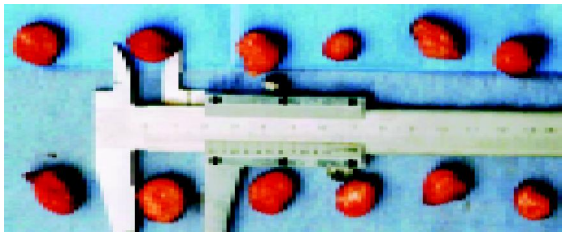

Figure 1 Tumors in nude mice on the 30th d. Upper line of tumors was HCC in the control group, the lower in therapy group.

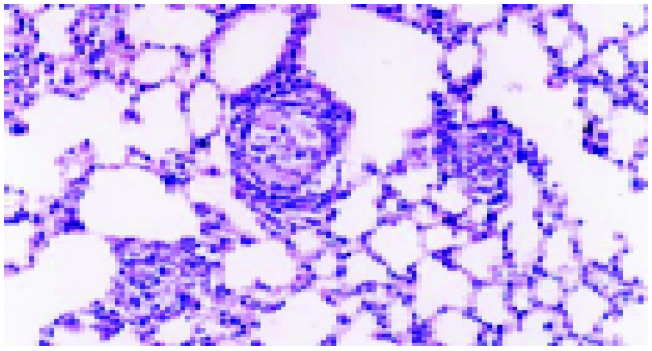

Figure 2 Metastases of hepatocellular carcinoma in lungs. Magnification: ×200.

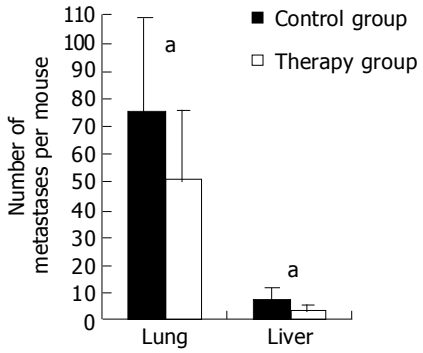

Figure 3 Metastases of hepatocellular carcinoma in lungs and liver. [a]$P<0.05$. Liver metastases were recorded in a gross manner by examining each lobe of liver and counting macroscopic tumors on the surface. Lung metastases were counted under microscope by observing consecutive paraffin slices of lung.

CD34 expression and microvessel counting

Expression of CD34 in therapy group was weak or even negative, whereas it was strong in control group. The newborn endothelial cells were stained brown or yellow and sinusoidly distributed in capillary walls of portal area and fiber interval of liver tissue (Figure 4). Microvessel counting revealed that MVD in control group was 80.00±26.27 per high-power field (×200), whereas it was 31.08±16.23 in therapy group ($P<0.05$).

Semi-quantitative RT-PCR

As shown in Figure 5, the PCR products of VEGF and G3PDH were visualized at the expected locations on agarose gels and in direct cDNA sequencing. All the obtained PCR products had the same cDNA sequences as the gene bank sequences (data not shown). The degree of VEGF mRNA by semi-quantitative RT-PCR was 0.0538±0.0165 in the therapy group, and 0.7373±0.1297 in the control group ($P<0.05$).

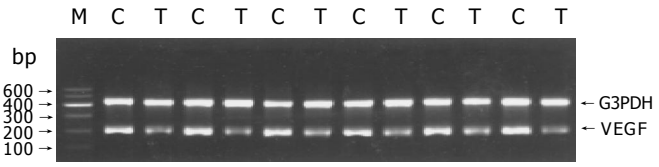

Figure 5 RT-PCR of VEGF mRNA in HCC tissue. M: Marker, 100 bp DNA ladder, ranging from 100 bp to 600 bp; C: Control group; T: Therapy group. The band of VEGF (all isoforms, 196 bp) and G3PDH (450 bp) are shown at expected location in the gel. G3PDH used as an internal standard. VEGF mRNA was expressed strongly in control group, whereas weakly in therapy group.

Effects on serum TNF-α and liver function indexes

Concentrations of serum ALT, AFP and TNF-α are shown in Table 2. The level of TNF-α in therapy group was lower than that in control group ($P<0.05$). No significant difference was observed in the in serum concentrations of ALT and AFP.

Table 2 Comparison of serum concentrations of ALT and AFP and TNF-α between groups (mean±SD)

Groups	ALT (IU/L)	AFP (μg/L)	TNF-α (ng/L)
Control (n = 12)	102.35±39.29	25.68±14.38	42.69±6.99
Therapy (n = 12)	87.88±35.38	19.40±13.58	28.64±4.64[a]

[a]$P<0.05$ *vs* control group.

DISCUSSION

Angiogenesis is a neovascularization process during which endothelial cells of the pre-existing capillaries proliferate and migrate to form new vascular tips or so called "vascular sprouts" or "endothelial buds". It is critical for the growth, invasion and

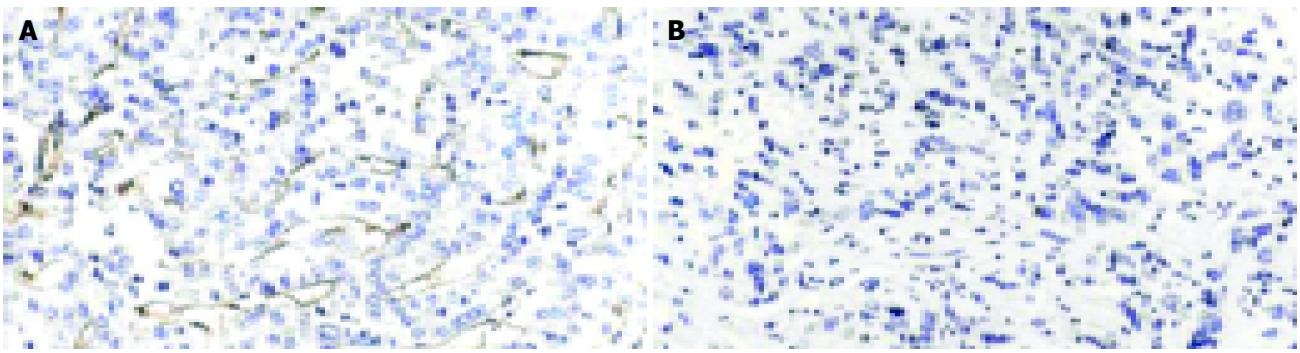

Figure 4 Immunohistochemical expression of CD34. A: control group; B: thalidomide treated group. Magnification: ×200.

metastasis of cancer[1,2,16]. Solid tumors would not grow beyond the volume of 2-3 mm^3 when sprouting new capillary blood vessels are tack, and the small cancer blocks, so-called micrometastases, would be kept in a hibernating state for a long term. Antiangiogenic therapy for malignant tumors has opened a brand new way to the treatment of carcinomas, and is regarded as one of the most promising and hopeful strategies. Different inhibitory effects of thalidomide on angiogenesis and tumor growth have been reported[17-19]. The current study was to examine the effect of thalidomide on angiogenesis and tumor growth and metastasis of human hepatocellular carcinoma.

In our study intraperitoneal administration was employed as previously described[14,15] at the dose of 200 mg/kg body weight every day. Kotoh *et al*[15] reported the antitumor and antiangiogenic effect of thalidomide on human esophageal cancer ES63 in nude mice by intraperitoneal administration. Whereas same effects were not observed when mice were treated by gavage administration. However, experiments *in vitro* have failed to demonstrate that thalidomide or any of its metabolites has any direct effect on cell proliferation or cytotoxic effect[20]. The mechanisms underlying the strong effect of antiangiogenesis by intraperitoneal route but poor efficiency by oral route remain unclear, the reason might be the bioavailability of the active form of thalidomide at tumor site. Results in this study reveal that changes of body weight and tumor weight and tumor volume in treatment group were smaller than those in control group, but statistical analysis showed no significant difference ($P>0.05$). Metastases in both liver and lung were observed. The metastatic rate in liver and lungs in treatment group and control group was 100%, but metastasis counting showed that the number of metastases in liver and lungs in treatment group was both statistically lower than those in control group ($P<0.05$). Minchinton *et al*[6] reported that thalidomide did not alter primary tumor growth of Lewis lung tumor xenoplanted in mice, and additionally reduced the radiosensitivity of the tumor, but did increase its sensitivity of combined treatment with radiation and cytotoxin tirapazamine. In another report[17], thalidomide alone inhibited tumor growth by 55% in the rabbit oral carcinoma model. However, Gutman *et al*[19] failed to find any antiangiogenic effect and tumor inhibition effect of thalidomide in syngeneic mice. Results reported are very different, it might be partly due to thalidomide's species specificity and tissue specificity[21]. Stephens *et al*[22] believe that various species and tissues depend on different angiogenesis or vascular pathways, the extent of dependence on integrin $\alpha_v\beta_3$ determines their sensitivity to thalidomide.

Angiogenesis is a highly complex and closely regulated process, which is influenced by the balance between stimulatory and inhibitory factors released by tumor and surrounding host cells. VEGF, bFGF and patelet-derived endothelial cell growth factor (PD-ECGF) are the main stimulatory factors, of which VEGF is the most important; it could promote the growth of malignant cells by increasing vascular permeability[23]. Researches have revealed that expression of VEGF in hepatocellular carcinoma is much higher than that in non-tumor tissues[24]. Concentration of VEGF in serum is also closely related to tumor's pathologic progress and patients' prognosis after operation[25]. In the current study we examined VEGF mRNA in cancer by semi-quantitative RT-PCR. It showed that the level of VEGF mRNA in thalidomide-treated group was significantly lower than that in control group. Also we immunohistochemically examined the expression of CD34 which was considered as an endothelial-specific marker, using monoclonal antimouse CD34 antibody. Results suggested that the expression of CD34 in therapy group was very weak, whereas in control group it was strongly expressed. Accordingly, MVD in the former was much less than that in the later ($P<0.05$). VEGF and MVD are the most common parameters reflecting neovascularisation. The effects of down-regulation in this study suggest thalidomide has inhibitory effects on angiogenesis. Its inhibitory effects on metastases in liver and lung might mainly attribute to its inhibition on VEGF[14], and its inhibition on integrin $\alpha_v\beta_3$ may also involve the process. Whether its obvious inhibition on metastasis but poor effects on tumor growth are due to its modulation on integrin $\alpha_v\beta_3$ is yet to be investigated.

The pharmaceutical role of thalidomide is very extensive, the most significant is to decrease the level of TNF-α in circulation so as to modulate immune system[26]. TNF-α, an important inflammatory factor, is also a critical factor to induce inflammatory reaction in hepatitis[27]. Raufman *et al*[28] reported a case of hepatitis C who's ALT in circulation was induced to normal by thalidomide. Serum TNF-α and liver function indexes such as ALT and AFP were tested in the study so as to try to find thalidomide's influence on liver function. Results suggest thalidomide could dramatically down-regulate serum TNF-α as previously reported[26], but no statistical variance was observed in serum concentration of ALT and AFP. Whether inhibition of TNF-α synthesis plays a different role in the inhibition of angiogenesis compared with immunomodulation has yet to be investigated. The remarkably inhibitory effect of thalidomide on TNF-α might be valuable for clinical treatment of liver cancer, because in China about 90% of hepatocellular carcinomas are accompanied with hepatitis B and abnormal liver function, the continuous inflammatory states of liver and liver failure after operation at least could partly contribute to the prognosis and poor life quality of patients. Although in this animal model hepatitis B virus genome was carried and AFP was expressed, its biological state of hepatitis might be very different from that of human beings. Further studies about the drug's effect on liver function are needed.

The molecular mechanisms and specific antitumor effects of thalidomide are yet to be elucidated, although some clinical trials have been performed[29]. Current clinical trials on thalidomide are mainly performed on unoperable malignant cases of middle or final phase, and in most cases tumor's volume change and disappearance are taken as standards to evaluate the drug's efficiency. Studies should be focused on elucidating the antitumor and antiangiogenic effects of thalidomide on specific cancers. Then in clinical trials this drug should be used as an adjunct treatment modality, its accumulating effects in long term and influence on the life quality of patients may be the most valuable, because thalidomide, as an antiangiogenic agent, does not have a remarkable dose-effect relationship as cytotoxic drugs. The results of this study suggest that thalidomide can be used as an adjunct treatment modality in the treatment of hepatocellular carcinoma.

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