Effect of thalidomide on the proliferation of hepatoma cells assessed by osteopontin levels in nude mice

FAN LIN¹, JIE CAO¹, ZHIMING HUANG², ZHENGHAO PEI¹, WEILI GU¹,
SHAOFENG FAN¹, KUNPING LI¹ and JIEFENG WENG¹

¹Department of General Surgery, The First People's Hospital Affiliated to Guangzhou Medical University, Guangzhou, Guangdong 510180; ²Department of General Surgery, Guangdong General Hospital, Guangzhou, Guangdong 510030, P.R. China

Received October 23, 2012; Accepted January 8, 2013

DOI: 10.3892/etm.2013.1010

Abstract. The aim of the present study was to investigate the inhibitory effects of thalidomide in the hepatocellular carcinoma nude mouse model in order to provide new insights into a comprehensive clinical intervention for hepatocellular carcinoma. MHCC97 cells were routinely cultured, passaged and adjusted to a single cell suspension with a concentration of 2x10⁶/ml. Six-week-old, BALB/C male nude mice were anesthetized and fixed in the prone position, then a subcapsular injection of the single cell suspension was administered into the spleen and their abdomens were closed. A laparotomy and left hepatic lobectomy was performed 14 days later and the abdomens were closed once again. Subsequent to the establishment of the hepatocellular carcinoma model, the nude mice were randomly divided into three groups, each consisting of 12 mice. The early intervention group were immediately provided with the post-operative thalidomide intervention, the late intervention group were provided with the post-operative thalidomide intervention one week subsequent to the surgery, and the negative control group were provided with a placebo intervention (0.9% physiological saline). Each intervention was continuously administered once per day for one week. The osteopontin (OPN) content of the liver tumors was detected using immunohistochemistry. The data were analyzed using an analysis of variance (ANOVA) test. There were significant differences in the OPN levels of the tumors among the early intervention, late intervention and negative control groups. Thalidomide may inhibit the generation of OPN and thereby inhibit the infiltration and metastasis of tumors; the immediate use of thalidomide following hepatectomy in the present study may block the invasion and metastasis for liver cancer more effectively.

Introduction

Cancer invasion and metastasis are difficult problems to overcome in cancer intervention. Genomics and transcriptional technology have been used to study the resected liver specimens of patients with hepatocellular carcinoma, as well as the molecular genetic features and gene expression profile in nude mouse and cell models of metastatic human hepatocellular carcinoma. It was identified that changes to the genes associated with liver metastasis occurred in the primary tumor stage and confirmed that osteopontin (OPN) had a significant predictive value and that it was the key transfer factor in hepatocellular carcinoma (1,2). This provided a new basis for the early diagnosis of hepatocellular carcinoma and for post-operative non-surgical intervention. These studies primarily answered the question of what invasion and metastasis of hepatocellular carcinoma are, but there have been few clinical studies concerning drug intervention in the invasion and metastasis of hepatocellular carcinoma. The present study aimed to evaluate whether thalidomide was able to inhibit the invasion and metastasis of hepatocellular carcinoma.

Thalidomide was first widely popularized and applied in West Germany in 1953 as a non-barbiturate sedative-hypnotic, mainly for the prevention of morning sickness. However, due to the teratogenic events (the side-effects were confined to pregnant women) associated with the drug, its use was forbidden in 1961. In 1991, D’Amato et al (3) identified that the teratogenic effect of thalidomide was related to the inhibition of new blood vessel formation. Subsequently, thalidomide was once more a focus of attention due to its effects in certain malignant tumors, particularly multiple myelomas. Thalidomide has also been identified to have extensive immune regulatory and anti-angiogenic effects. In 1998, thalidomide was approved by the FDA for use in clinical trials. There have been worldwide studies on thalidomide intervention in malignant tumors, however this cheap and well-known drug has commonly been limited in its use due to its unknown mechanism of action and the lack of support from evidence-based medical studies (4-7).

Thalidomide may act via a series of cascading effects with OPN involving new cell signaling pathways or media to control the expression and molecular behavior of intercellular substances in the hepatocellular carcinoma tumor microenvi-
rnonment, and thereby directly or indirectly repress the invasion and metastasis of hepatocellular carcinoma. The elucidation of its mechanism may facilitate significant improvements in structure-activity studies of thalidomide and promote its use in tumor translational medicine.

Materials and methods

General data. A total of 36 BALB/C male nude mice (aged 6-7 weeks old), were purchased from the Laboratory Animal Center of the Medical College of Guangzhou Medical University. The MHCC97 cells were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The present study was carried out in strict accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol for animal use was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First People's Hospital Affiliated to Guangzhou Medical University (Guangzhou, China).

Establishment of the nude mouse model of hepatocellular carcinoma. Following successful anesthesia, the nude mice were fixed in the prone position and conventional skin disinfection was performed. A 1-cm straight incision was made next to the spine 0.5 cm from the left costal margin, descending layer by layer into the abdomen. The incision was bluntly drawn to expose the spleen and then 0.1 ml MHCC97 single cell suspension was drawn up into a 1-ml insulin syringe and injected 0.3-0.4 cm into the spleen. Subsequent to being injected with 0.02 ml (~8×10⁶ cells per mouse), the syringe was quickly withdrawn. The conditions of the mice were observed, including whether or not there was bleeding or intestinal oppression in the spleen and whether there was any contortion in the intestinal tract or mesentery. The abdomen was then closed layer by layer using a needle and 5-0 sutures.

Partial hepatectomy and post-operative management of the nude mice. A partial hepatectomy of the liver was performed 14 days after splenic subcapsular inoculation with the MHCC97 cells. The nude mice were fed separately following xenografting of the hepatocellular carcinoma model, the nude mice were randomly divided into three groups, each consisting of 12 mice. Subsequent to the partial hepatectomy, the early intervention group were immediately injected with a placebo of 0.1 ml 0.9% physiological saline into the tail vein as the replacement intervention. Continuous administration was also provided for one week. The mice were sacrificed immediately prior to dissection. The liver tumor tissues were dissected, separated and fixed in formalin 21 days after the partial heptectomy. The OPN contents of the liver tumors and paracarcinomatous tissues were detected using immunohistochemistry.

Calculation of results. The results of the staining process were judged using Bresalier semi-quantitative formulae. A total of 10 fields of view were randomly selected in each slice using high magnification (x200) and the double-blind method. The fields of view were divided into four grades according to the cell staining intensity and then scored as follows. Negative cells (-) showed no coloration (0 point); weakly positive cells (+) were light yellow (1 point); moderately positive cells (++) were claybank in color (2 points); and strongly positive cells (++++) were brown (3 points). The number of fields of view of each strength were counted and the average stain strength of each slice was calculated according to the following formula: IS (intensity score) = \( \sum [(0 \times F0) + (1 \times F1) + (2 \times F2) + (3 \times F3)] \), F = % x 10 fields of view.

Statistical analysis. Statistical analyses were performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA). The data are presented as mean ± SD and a t-test and one-way analysis of variance (ANOVA) was used for the comparison of the data between the groups. P<0.0091 was considered to indicate a statistically significant result.

Results

HE staining. Yellow-white nodules were present in the livers of all 36 experimental animals that underwent partial hepatectomy subsequent to being sacrificed. The specimens of liver tumor tissue in all 36 cases were identified to be hepatic carcinoma following HE staining (Fig. 1).

Immunohistochemistry results. As shown in Fig. 2, the OPN-positive cells in the tumor tissues of the early inter-
The OPN content in the pericarcinomatous liver cells in the late intervention group was lower than that in the tumor tissues. Immunohistochemistry results (qualitative determination). The OPN level in the tumor tissues of the early intervention group (1.079±0.345) was significantly lower than that of the negative control group (2.775±0.094; Table I: \( F=269.57, P<0.05 \)). The OPN level in the tumor tissues of the late intervention group (1.898±0.342) was also significantly lower than that of the early intervention group (1.079±0.345; Table III: \( F=34.12, P<0.05 \)).

In the negative control group without thalidomide treatment, OPN (2.775±0.094) was highly expressed in the hepatocellular carcinoma tissues and was at a significantly higher level than that in the pericarcinomatous tissues (Table IV: \( F=328.74, P<0.05 \)).

Discussion

OPN is a secretory phosphorylated glycoprotein, with a relative molecular mass of ~44 kDa, containing ~300 amino acid residues, of which aspartic acid, serine and glutamic acid residues account for a high proportion. It has been demonstrated that OPN is largely synthesized and secreted in malignant tumor cells, particularly in hepatocellular carcinoma (1,2).
intramolecular structure, RGD (Arg-Gly-Asp), is an unique sequence that improves cell adhesion in proteins. Through the RGD cell adhesion sequence, OPN may interact with important tumor metastasis factors, including integrin, CD44, vascular endothelial growth factor/epidermal growth factor receptor (VEGF/EGFR), matrix metalloproteinases (MMPs), fibronectin (FN), survivin, transforming growth factor (TGF), tumor necrosis factor (TNF) and urokinase-type plasminogen activator (uPA) to promote cell chemotaxis, adhesion and migration (8-17). Budhu et al (18) and Pan et al (19) put forward the theory that OPN was a significant factor in hepatocellular carcinoma metastasis and that it may be a molecular marker of intrahepatic metastasis. These authors also suggested that OPN may act via the PI3K/NF-κB cell signaling pathway. In our preliminary studies (3,20,21), the excessive expression of OPN was was identified to be closely correlated with the early metastasis and relapse of hepatocellular carcinoma, which is a significant factor in a poor prognosis following hepatectomy. OPN had varying expression levels in hepatoma cells with different metastatic potentials and was not expressed in normal hepatic cells. These differences were statistically significant. OPN was confirmed to have utility as a sensitive index for predicting micro-metastases in early hepatocellular carcinoma. OPN may be considered as a bridge connecting primary and metastatic tumors in hepatocellular carcinoma.

It has been reported (22) that thalidomide prevents basic fibroblast growth factor (bFGF) and VEGF from inducing angiogenesis, which may involve multiple pathways. However, the specific mechanism by which thalidomide inhibits angiogenesis remains unclear. We have previously attempted to use thalidomide intervention in 7 patients with unexplained, permanent hematochezia in the clinic and observed unexpected effects. We speculated that thalidomide may be related to uPA (23). Thalidomide may block the blood flow to malignant tumors and the resultant lack of nutrition may then reduce the growth of tumor cells and cause them to atrophy, thereby extending the lives of affected patients. Matrix metalloproteinase-9 (MMP-9) is a significant member of the MMP family, with the largest molecular weight (92 kDa). MMP-9 decomposes the extracellular matrix (ECM) and is involved in a number of physiological and pathological processes in the human body. Collagen types IV, V, VII and X, gelatins and elastin fibers are its main substrates. MMPs degenerate the basement membrane barrier and act with various cytokines to promote the formation of new blood vessels in tumors and the proliferation of tumor cells (24). MMPs may also participate in evasion of the host's immune surveillance to promote the growth of tumors, as well as participating in invasion and metastasis. We speculate that thalidomide may affect the activity and expression of VEGF to reduce the stimulation of metastatic potentials and was not expressed in normal hepatic cells. These differences were statistically significant. OPN was confirmed to have utility as a sensitive index for predicting micro-metastases in early hepatocellular carcinoma. OPN may be considered as a bridge connecting primary and metastatic tumors in hepatocellular carcinoma.

In conclusion, as an older drug with a mature production line, thalidomide is cheap and convenient to use. The specific mechanism of thalidomide requires clarification if it is intended to be comprehensively used as a clinical antitumor drug or to replace or be used in combination with expensive new drugs. This would assist in correcting the historical prejudices against thalidomide. Previous studies into thalidomide have shown that it plays a significant role in the treatment of various difficult and severe diseases. With the continuous development
of clinical and pharmacological studies, the effects of thalidomide and its mechanism of action may become clearer and better defined. The present study supports the positive effect of thalidomide as an anticancer treatment that is cheap. The present results show that thalidomide prohibits the liver cancer as it targets osteopontin.

References

1. Tang ZY, Ye SL, Liu YK, et al: A decade's studies on metastasis of hepatocellular carcinoma. J Cancer Res Clin Oncol 130: 187-196, 2004.
2. Qin LX and Tang ZY: Recent progress in predictive biomarkers for metastatic recurrence of human hepatocellular carcinoma: a review of the literature. J Cancer Res Clin Oncol 130: 497-513, 2004.
3. D'Amato RJ, Loughnan MS, Flynn E and Folkman J: Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91: 4082-4085, 1994.
4. Shortt J, Hsu AK and Johnstone RW: Thalidomide-analogue biology: immunological, molecular and epigenetic targets in cancer therapy. Oncogene: Jan 14, 2013 [Epub ahead of print].
5. Chen YY, Yen HH, Chou KC and Wu SS: Thalidomide-based multidisciplinary treatment for patients with advanced hepatocellular carcinoma: a retrospective analysis. World J Gastroenterol 18: 466-471, 2012.
6. Ang SF, Tan SH, Toh HC, Poon DY, Ong SY, Foo KF and Choo SP: Activity of thalidomide and capcitabine in patients with advanced hepatocellular carcinoma. Am J Clin Oncol 35: 222-227, 2012.
7. Garrison LJ Jr, Wang ST, Huang H, et al: The cost-effectiveness of initial treatment of multiple myeloma in the u.s. With bortezomib plus melphalan and prednisone versus thalidomide plus melphalan and prednisone or lenalidomide plus melphalan and prednisone with continuous lenalidomide maintenance treatment. Oncologist 18: 27-36, 2013.
8. Ue T, Yokozaki H, Kitadai Y, et al: Co-expression of osteopontin and CD44v9 in gastric cancer. Int J Cancer 79:127-132, 1998.
9. Wallach RC: Osteopontin as a biomarker for ovarian cancer. JAMA 287: 3209-3210, 2002.
10. Robinson BW and Lake RA: Advances in malignant mesothelioma. N Engl J Med 353: 1591-1603, 2005.
11. Wai PY and Kuo PC: The role of Osteopontin in tumor metastasis. J Surg Res 121: 228-241, 2004.
12. Teramoto H, Castellone MD, Malek RL, et al: Autocrine activation of an osteopontin-CD44-Rac pathway enhances invasion and transformation by H-RasV12. Oncogene 24: 489-501, 2005.