Experimental study of thalidomide for treatment of murine hepatocellular carcinoma

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Abstract: Objective To study the therapeutic effect of thalidomide (Tha) on murine hepatocellular carcinoma. Methods In murine transplanted hepatoma model, thalidomide was administered intragastrically alone (200 mg/kg daily for 10 days) or in combination with doxorubicin. The antitumor activity of Tha was observed in solid and ascitic tumor models. Results Tha induced significant growth inhibition of solid hepatoma without obvious toxicity on peripheral blood cells and lymphocyte proliferation. Although Tha alone had no effect on the survival of mice with ascitic tumor, it showed a synergistic antitumor activity in combination with doxorubicin (Dox) in both solid and ascitic tumor models. Moreover, Tha reduced Dox-induced cytopenia and immunosuppression. Histological analysis of Tha-treated tumors revealed remarkably enhanced tumor necrosis and lymphocyte infiltration on the edge of tumor tissues. Conclusion Tha has definite therapeutic effect on murine hepatoma, and the combination with Dox shows an enhanced therapeutic potential.

Key words: thalidomide; hepatocellular carcinoma, solid/ascitic; doxorubicin

Thalidomide (Tha) had been used as a sedative and antiemetic in the 1950s, but subsequently banned for clinical use due to its severe side effect of teratogenicity [1]. Recent studies have shown that Tha possesses significant antitumor activity in animal models and some clinical trials [2]. Tha was reported to produce inhibitory effect on the proliferation of hepatomatal cell line in vitro [3], which shed light on treatment of human hepatocellular carcinoma for which currently no effective therapeutic approaches are available. The aim of this study was to evaluate the therapeutic efficacy of Tha in the treatment of murine hepatocellular carcinoma.

MATERIALS AND METHODS

Drugs and reagents

Tha (supplied by Jiangsu Changzhou Pharmaceutical Factory) was dissolved in sterile water at appropriate concentration before use. RPMI 1640, MTT, concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma Co. Fetal bovine serum (FBS) was purchased from Baiye Biological Products Company (Tianjin, China). penicillin and streptomycin were obtained from North China Pharmaceutical Group Corporation. Blood cell count kit was obtained from Institute of Biological Products of Henan Province. All other reagents were of analytic grade.

Animals and cell line

Kunming strain mice (18-22 g, either sex) were purchased from Experimental Animal Center of Henan Province and kept in the laboratory for one week before the experiments. The mice were housed under standard conditions on a 12 h light/dark cycle with free access to food and water. Mouse HepA hepatocarcinoma cell line was kindly provided by the Laboratory of Cytology, College of Pharmacy, Henan University.

Instruments

BioRad microplate reader 550, NAPCO CO2 incubator, and Sorvall RT7 centrifuge were used in this study.

Tumor model establishment [4]

HepA cells were collected from the ascites of mice 8 days after HepA cell inoculation and passaged routinely to prepared cell suspension of 5 x 10^6/ml. Kunming mice received a subcutaneous injection of HepA cells (1 x 10^6 cells in 0.2 ml) in the right axillary region to induce the growth of solid tumor, or a peritoneal injection to establish mouse models of ascitic tumor.

Grouping and drug administration

The mice with HepA cell implantation were randomly divided into 4 groups (n=30 each), namely the normal control group receiving intragastric administration of saline, Tha group with intragastric Tha administration at the daily dose of 200 mg/kg, Dox group with intraperitoneal Dox injection at the daily dose of 4 mg/kg, and Tha+Dox group with administra-
tion of both Tha and Dox in the same manner as specified. Tha was administered for 10 days and Dox every 2 days for a total of 3 doses.

**Response evaluation**

For ascitic tumor model, the survival time was recorded. The observation of tumor development lasted for 60 days and the mice surviving for more than 60 days were recorded to have survived for 60 days. The survival prolongation rate and the coefficient of concordance were calculated as follows:

Survival prolongation rate = (mean survival in treatment group/mean survival in control group − 1) × 100%,

Coefficient of concordance = (mean survival in Tha group × mean survival in Dox group)/ (mean survival in control group × mean survival in Tha + Dox group).

The survival prolongation was considered significant when the prolongation rate > 75% and P < 0.05, and the synergistic effect was considered significant when the coefficient of concordance < 1.

For solid tumor model, the body weight of the mice was measured and blood collected via orbital sinus puncture on day 11 of treatment. Then all the mice were sacrificed and the spleens and tumors were excised for lymphocyte proliferation assay and histopathological examinations, respectively. The tumor growth inhibition rate and the coefficient of concordance were calculated using the following formulas:

Tumor growth inhibition rate = (1 − mean tumor weight in treatment group/mean tumor weight in control group) × 100%

Coefficient of concordance = mean tumor weight in control group/mean tumor weight in Tha + Dox group / mean tumor weight in Tha group/mean tumor weight in Dox group

The antitumor activity was considered to be significant when the inhibition rate > 40% and P < 0.05; the synergistic effect was considered significant when the coefficient of concordance < 1.

**Routine blood test**

The assay was performed using the blood cell count kit according to the manufacturer's instructions.

**Lymphocytes proliferation assay**

The spleen was homogenized with Hank's balanced salt solution and 0.83% Tris-NH₄Cl was used to lyse the erythrocytes. The splenocytes were resuspended at the concentration of 1×10⁶ cells/ml in RPMI1640 medium supplemented with 10% FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin. The splenocytes of 200 ml were seeded into 96-well plates in the presence of 5 mg/ml ConA and incubated in 5% CO₂ incubator at 37 °C for 72 h (6 wells for each group). MTT solution (5 mg/ml, 20 ml) was then added followed by further incubation for 4 h. The supernatant was removed from the plates after centrifugation at 1000 r/min for 5 min. Subsequently, 100 ml DMSO was added and shaken for 2 min, and finally the plates were read at 570 nm on BioRad microplate reader 550.

**Statistical analysis**

All data are presented as Mean±SD. Comparisons were made using one-way ANOVA, with a P value less than 0.05 considered to denote significant statistical difference.

**RESULTS**

**Effect of Tha alone or combined with Dox on murine hepatocellular carcinoma**

Tha treatment alone at the dose of 200 mg/kg for 10 days produced prominent growth inhibition of the solid tumors, but had no effect on the overall survival of the mice bearing ascitic tumors. However, Tha combined with Dox led to synergistic antitumor effect on both solid and ascitic tumors (Tab.1).

<p>| Tab.1 Growth inhibition of mouse hepatocellular carcinoma induced by Tha alone or in combination with Dox (n=30) |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (mg/kg× times)</th>
<th>Solid tumor inhibition rate (%)</th>
<th>Survival prolongation rate of mice with ascitic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>2.38±0.41 (tumor weight)</td>
<td>12.3±2.4 (life span)</td>
</tr>
<tr>
<td>Tha</td>
<td>200×10</td>
<td>42.31**</td>
<td>8.90</td>
</tr>
<tr>
<td>Dox</td>
<td>4×3</td>
<td>53.85**</td>
<td>118.49**</td>
</tr>
<tr>
<td>Tha/Dox</td>
<td>200×10/4×3</td>
<td>74.62**</td>
<td>162.33**</td>
</tr>
</tbody>
</table>

**P<0.01 vs control group. The concordance coefficient between Tha and Dox was 0.953 in solid tumors and 0.907 in ascitic tumors**

**Effect of Tha on blood cell count and lymphocyte proliferation in mice bearing solid hepatocellular carcinoma**

Tha treatment alone significantly inhibited the growth of solid hepatocellular carcinoma, but did not reduce the blood cell count or inhibit lymphocyte proliferation. When combined with Dox, Tha partly prevented the decrease of peripheral blood cells as well as immunosuppression induced by Dox (Tab.2).
Histological evaluation of hepatocellular carcinoma in mice treated with Tha

Compared with the control group, Tha significantly promoted tumor tissue necrosis as well as lymphocyte infiltration on the edge of the tumor tissues (Fig.1).

![Histological Images](image)

**Fig.1 Pathological evaluation of hepatocellular carcinoma in mice treated with Tha**

A1: Morphology in the control group; A2: Lymphocyte infiltration in control group; B1: Morphology in Tha group; B2: Lymphocyte infiltration in Tha group; C: Morphology in doxorubicin group; D: Morphology in Tha+Dox group

DISCUSSION

Tha, the once culprit drug for causing phocomelia in over ten thousands of newborns, has reentered into clinical use after discovery of its wide immunomodulatory and antiangiogenic capabilities. More recently, there has been great clinical interest in Tha for its potential in treating malignant tumors. Although its exact antitumor mechanisms are still unclear, its antiangiogenic activity has been thought to be mainly responsible for its antitumor effects. However, it was also reported that Tha inhibited tumor growth through both antiangiogenesis and direct cytotoxicity against tumor cells. In the present study, Tha treatment alone significantly inhibited tumor growth of implanted solid hepatocellular carcinoma, but had no effect on the overall survival of mice with ascitic hepatoma. The combination of Tha and Dox resulted in synergistic antitumor activity in both solid and ascitic tumor models, without producing obvious toxicity on the blood cells and lymphocyte proliferation. Our results provided the experimental basis for treatment of hepatocellular carcinoma with Tha, and suggested that the antitumor activity of Tha may not be completely attributed to direct cytotoxicity against tumor cells.

Tha was reported to have potent immunomodulatory and antiangiogenic activities, and might be able to enhance the effectiveness and decrease the toxicity of chemotherapeutic drugs. We investigated the effect of Tha in combination with Dox against mouse liver cancer and found that their combination exerted a synergistic antitumor activity on both solid and ascitic hepatocellular carcinoma. Moreover, Tha partly prevented the toxicity of cytopenia and immunosuppression induced by Dox, and significantly promoted tumor tissue necrosis as well as lymphocyte infiltration on the edge of the tumor tissues (Fig.1).
increased necrosis and lymphocyte infil- tration of the tumor tissues at the dose of 200 mg/kg. In accordance with our hypothesis, the present study identified definite therapeutic efficacy of Tha on mouse liver cancer and an enhanced antitumor activity in combination with Dox. In addition, no significant reduction in body weight or other toxicities were observed throughout the treatment. In view of the fact that Tha has been approved for the treatment of patients with multiple myeloma in USA and some European countries, we suggest that the clinical trial of Tha be considered in human hepatocellular carcinoma. If the clinical data is consistent with the data obtained from animal models, Tha will provide a novel and potential strategy for treatment of human hepatocellular carcinoma.

REFERENCES


沙利度胺治疗肝癌的实验研究

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摘要: 目的 研究沙利度胺对肝癌的治疗作用。方法 采用小鼠肝癌移植性模型, 观察沙利度胺对实体型和腹水型肿瘤的治疗作用。结果 沙利度胺按每日 200 mg/kg 连续给药 10 d, 能明显抑制肝癌实体型肿瘤的生长, 不降低小鼠血细胞数及淋巴细胞增殖; 对腹水型肿瘤小鼠虽然减轻生命延长作用, 但沙利度胺与阿霉素联合用药对肝癌实体型及腹水型均有协同抗肿瘤作用, 且能阻止阿霉素造成的小鼠血细胞减少, 免疫功能降低。沙利度胺日剂量 200 mg/kg 能明显增加肿瘤组织坏死, 促进肿瘤组织边缘淋巴细胞侵润。结论 沙利度胺对小鼠肝癌有确切治疗作用, 与阿霉素联合用药效果更好。关键词: 沙利度胺; 肝癌, 实体型; 腹水型; 阿霉素