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A PRELIMINARY STUDY OF THE ANTI-CANCER EFFECT OF TANSHINONE ON HEPATIC CANCER AND ITS MECHANISM OF ACTION IN MICE

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Objective: There were some experimental researches *in vitro*, which showed that tanshinonoe (Tan) had cytotoxic activities against some cancer cell lines. But there was no report of anti-cancer activity of Tan *in vivo*. This experimental study was performed to confirm the anti-cancer activity of Tan *in vivo*. Methods: Hepatic carcinoma H_{22} bearing mice were treated with DMSO, 5-Fu, and Tan, at the end of experiment, the mice were sacrificed, tumor tissues were separated and weighed, and the tumor inhibitory rate was calculated, 3 times of the same experiments were performed. The proliferating kinetics of hepatic carcinoma H_{22} cells in mice was measured by bromodeoxyuridine labeling *in vivo* and immunohistochemical staining of the proliferating cell nuclear antigen (PCNA) in tumor tissues. Results: The tumor inhibitory rates of Tan were 50.0%, 38.5%, and 40.6% in 3 experiments, respectively, compared with those of the DMSO-treated control groups, the differences were significant statistically ($P<0.01$). The Brdu labeling and PCNA positive cells were 51.8± 7.9 and 451.1± 26.1, respectively, which were significantly lower than those of controls (84.4± 24.3, 694.8±117.1) ($P<0.01$). Conclusion: Tan had anti-cancer effect on hepatic carcinoma *in vivo*; The mechanisms of action might be associated with inhibition of DNA synthesis, PCNA expression and DNA polymerase δ activity of tumor cells.

Key words: Tanshinone, Anti-cancer effect, Hepatic neoplasms

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Tanshinone is the effective component extracted from Chinese medical herb, Radix Salviae Miltiorrhizae.[1,2] The experimental research *in vitro* indicated that Tanshinone had obvious inhibiting effect on DNA synthesis of cancer cells and cytotoxic activities against some human cancer cell lines,[2,3] and also had effect of inducing differentiation on human cervical cancer cell line.[4] But, there is no report of the anti-cancer effect of Tanshinone *in vivo* and studies of its mechanism of action. In order to confirm the anti-cancer effect of Tanshinone and to study its mechanism of action, this experimental research *in vivo* was performed, which may provide some scientific data for the clinical study and usage.

MATERIALS AND METHODS

Tumor Inhibiting Experiment in Mice

Animals, NIH mice, body weight 20-24g, both females and males were provided by the Experimental Animal Laboratory of Sichuan Antibiotic Industrial Institute (Certificate number, No. 67). More than 10 mice with the same sex were used in each experiment. Hepatic cancer cell line H_{22} was provided by Institute of Cancer Research, WCUMS. Tanshinone (Tan IIA) was provided Chinese Institute for Drug and Biological Product Control (Lot number, 766-9204), before its usage, Tan was diluted to required

concentration with 10% DMSO. Fluorouracil injection (5-Fu) was manufactured by Tianjing Hebei General Pharmaceutieal Factory (Lot number 931119), before its usage, 5-Fu was diluted with physiological saline. The ascites of mouse bearing hepatic cancer H_{22} was taken and diluted with physiological saline to a concentration of 1×10^7/ml cancer cells. 0.2 ml of cancer cell solution was injected s.c. to each mouse. In the second day of the tumor inoculation, the tumor bearing mice were grouped to 3 groups at random. i.e. The negative control, in which 0.2 ml DMSO was injected s.c.; The positive control, in which 5-Fu at a dosage of 30 mg/kg (body weight) was injected i.p.; The test group, in which Tan at a dosage of 20 mg/kg was injected s.c.; The positive control, in which 5-Fu at a dosage of 30mg/kg (body weight) was injected i.p.; The test group, in which Tan at a dosage of 20 mg/kg was injected s.c.. Totally, 3 times of the same experiments were performed. The animal numbers and administration times of each group were showed in Table1. In the second day of the end of the administration in each experiment, the animals were sacrificed; the tumor tissues were separated and weighed; the tumor inhibitory rates were calculated and analyzed with the student's test statistically. The tumor tissues of each group were fixed in 20% formalin. Paraffine sections and HE staining were made, and observed under light microscope.

Table 1. The inhibiting effect of Tan on hepatic cancer in mice

No.	Treat	Dosage ($mg.kg^{-1}.d^{-1}$)	Ways & times	No. of animals		Body weight (g)	Average tumor wt. $\bar{\chi}\pm s$(g)	Tumor inhibiting rate(%)	*P*
				before	end				
1	DMSO	10ml	scx5	10	10	+2	1.00± 0.31		
	5-FU	30mg	ipx4	11	11	-0.5	0.44± 0.34	56.0	<0.01
	Tan	20mg	scx5	10	10	+7	0.50± 0.38	50.0	<0.01
2	DMSO	10ml	scx5	14	14	+ 0.5	1.69± 0.50		
	5-Fu	30mg	ipx4	14	14	+ 3.5	0.91± 0.27	46.2	<0.01
	Tan	20mg	scx5	12	12	+ 3.1	1.04± 0.47	38.5	<0.01
3	DMSO	10ml	scx5	16	16	+ 4.2	1.06± 0.38		
	5-Fu	30mg	ipx4	16	16	+ 2.0	0.48± 0.36	54.7	<0.01
	Tan	20mg	scx5	12	12	+ 4.5	0.63± 0.33	40.6	<0.01

* sc: subcutaneous injection; ip: intraperitioneal injection

Brdu Incorporating and Detecting

5-bromo-2'-deoxy-uridine and detection kit II (Boerhringer Mannheim Biochemica, Germany) was used in this method. In the third experiment, 0.2 ml of Brdu labeling solution diluted with physiological saline at 1:5 was injected i.p. to each mouse. 4 hours late, tumor tissues were taken and fixed in 70% ethanol-glycin fixative; paraffine sections were made and stained with immunocytochemistry method to detect the Brdu labeled tumor cells.[5] The areas of well grown tumor tissues with little inflammatory cell infiltration, without bleeding and necrosis, and more labeled cells were taken as observing fields under low power lens. Under middle power lens (×400), the labeled cells in 5 fields were counted for each case (total cell number >2000). And the results were analyzed with Chi-square test statistically.

Detecting Proliferating Cell Nuclear Antigen (PCNA) with Immunohistochemistry Method

In this method, PC10 McAb against PCNA (DAKO) and S-P kit (Maxim Co. U.S.A.) were used. Immunohistochemical staining was performed with LSAB method according to the introduction of S-P kit with a little modification. PC10 was diluted at a concentration of 1: 200, human tonsil tissue was taken for positive control; And PBS was used instead of PC10 for negative control. The observing method was the same as the method of Brdu labeled cell observation, but the PCNA positive cells in 10 high power fields (× 1000) were counted (total cell number >2000). The results were analyzed with Chi-square

test statistically.

RESULTS

The inhibitory Effect on Hepatic Carcinoma H_{22} of Tan in mice

The tumor inhibitory rates of Tan of 3 experiments were 50.0%, 38.5%, and 40.6%, respectively ($P<0.01$). And no differences of body weight and general condition were observed in the test group compared with the negative control.

Histopathological Observation

The different grades of karyopyknosis, necrosis of cancer cells, bleeding, and inflammatory cell infiltration were observed in the Tan treated groups, and severer than those of the control groups.

The Number of Brdu Labeled Cells and PCNA Positive Cells

Both the number of Brdu labeled cells and the number of PCNA positive cells in tumor tissues of Tan treated groups were significantly lower than those of the control groups ($P<0.01$, Table 2).

Table 2. The number of Brdu labeled and PCNA positive cells the tumor tissues of each group

Treat group	No. of animal	Brdu labeled cells ($\bar{\chi} \pm s$)	P	PCNA positive cells ($\bar{\chi} \pm s$)	P
DMSO	12	84.4± 24.3		694.8± 117.1	
5-Fu	12	27.8± 3.9	<0.01	471.9± 65.9	<0.01
Tan	11	51.8± 7.9	<0.01	451.1± 26.1	<0.01

DISCUSSION

The Anti-cancer Effect of Tan

Tan was a kind of fat-soluble component extracted from Chinese medical herb, Radix Salviae Miltiorrhizae, it could enhance the effect on the anti-cancer activities of camptothecine and cyclophosohamide to Ehrlich ascites cancer, hepatic cancer, sarcoma S-180, leukemia L615.[1] The experiment *in vitro* showed that Tan had anti-cancer effect on ascites cancer,[1,2] cytotoxic activities against some human cancer cell lines,[3] and effect of inducing differentiation on human cervical cancer cell line.[4] However, there was no report about the anti-cancer activity of Tan *in vivo*. In this experimental research, the tumor inhibitory experiment of Tan *in vivo* against hepatic cancer H_{22} in mice was performed. The results indicated that Tan had anti-cancer effect on hepatic cancer *in vivo* and without obvious toxic and side effect. It is worthy of further experimental study and usage clinically.

The Anti-cancer Mechanism of Tan

There had already been the report on the tumor inhibiting and anti-cancer mechanism of Tan. Chang, et al.[2] studied the anti-cancer activity of Tan with IudR incorporating method, indicated that sarcoma S-180 cell line cultured *in vitro* acted with Tan, the IudR incorporating rate of cancer cells was obviously lower than that of the control. And he thought Tan acted on the S phase of cell cycle by inhibiting the synthesis of DNA and had cytotoxic activity. Yuan, et al.[4] reported that human cervical cancer cell line ME180 after having been acted with Tan, ^{3}H-TdR incorporating rate was significantly lower than that of controls, which indicated that Tan had the effect on inhibiting the proliferating of tumor stem cells and DNA synthesis. Connolly, et al.[6] discovered that cells in late S phase can be labeled with Brdu. Brdu incorporating method had an applied value in the studies of tumor chemotherapy and mechanism of carcinogenesis. Teodori, et al.[7] reported that Brdu labeling indexs in the tumors with the double time (DT)≥90 days were significantly lower than those in the tumors with DT≤90 days. It was believed that Brdu index expressed the growth state and biological characteristics of tumor *in vivo*. In this experimental

research, the average tumor weight and Brdu labeled cells in tumor tissues of animals treated with Tan were significantly lower than those in the controls. It was indicated that tumor cells after having been treated with Tan in vivo, the amount of DNA synthesis decreased, the number of cells entering S phase decreased. Thereby, the growth of tumor was inhibited. This result held identical views in the researches *in vitro* of the other investigators.

PCNA is an auxiliary protein of DNA polymerase δ, which begins to increase in late G1 phase in cell cycle and reach to a peak in S phase. It plays a role in DNA replication and in the process of cell proliferation. PCNA expression and its amount reflect the proliferating activity of cells.[8] To measure the proliferating activity of cancer cells before and after the treatment of cancer patients could be taken as an indicator to judge the reaction of tumor to the chemotherapy, which therefore could provide scientific basis for clinical treatment of cancer patients.[9] In this experimental research, PC10 McAb was used to detect PCNA positive cells in the tumor tissues of each group by the method of immunohistochemical staining. PCNA positive cells represented the cells in the late G1 phase and early S phase of cell cycle. The number of PCNA positive cells in tumor tissues of Tan treated group (445.1± 26.1) was lower than that of control group (694.8± 117.1), significant difference was found statistically ($P<0.01$). Based on the results of this experimental research, it can be inferred that the tumor inhibiting and anti-cancer mechanisms of Tan might be associated with inhibition of expression and increase of PCNA, and with inhibition of the activity of DNA polymerase δ of tumor cells. Thereby, the DNA synthesis of tumor cells was inhibited, cells entering into S phase decreased, then, the growth of tumors slowed up. In addition, the histopathological observation of karyopyknosis, degeneration, necrosis, and diffused inflammatory cell infiltration in the tumor tissues of the animals treated with Tan might be associated with enhancement of immune function of body or the other mechanisms of cytotoxic activities acting on cancer cells. Therefore, the anti-cancer activity of Tan and the mechanisms of its action deserve further studies.

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