• BRIEF REPORTS •

Effect of resveratrol on cell cycle proteins in murine transplantable liver cancer

Liang Yu, Zhong-Jie Sun, Sheng-Li Wu, Cheng-En Pan

Liang Yu, Zhong-Jie Sun, Sheng-Li Wu, Cheng-En Pan, Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China
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Correspondence to: Dr. Liang Yu, Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China. yuliang@163.com
Telephone: +86-29-5324009
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Abstract

AIM: To study the antitumour activity of resveratrol and its effect on the expression of cell cycle proteins including cyclin D1, cyclin B1 and p34cdc2 in transplanted liver cancer of murine.

METHODS: Murine transplanted hepatoma H22 model was used to evaluate the *in vivo* antitumor activity of resveratrol. Following abdominal administration of resveratrol, the change in tumour size was recorded and the protein expression of cyclin D1, cyclin B1 and p34cdc2 in the tumor and adjacent noncancerous liver tissues were measured by immunohistochemistry.

RESULTS: Following treatment of H22 tumour bearing mice with resveratrol at 10 or 15 mg/kg bodyweight for 10 days, the growth of murine transplantable liver cancer was inhibited by 36.3 % or 49.3 %, respectively. The inhibitory effect was significant compared to that in control group ($P<0.05$). The level of expression of cyclin B1 and p34cdc2 protein was decreased in the transplantable murine hepatoma 22 treated with resveratrol whereas the expression of cyclin D1 protein did not change.

CONCLUSION: Resveratrol exhibits anti-tumour activities on murine hepatoma H22. The underlying anti-tumour mechanism of resveratrol might involve the inhibition of the cell cycle progression by decreasing the expression of cyclinB1 and p34cdc2 protein.

INTRODUCTION

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a kind of phytoalexin found in root extract of the weed polygonum cuaspidatum and in grape skins as well as red wine. Studies have demonstrated that resveratrol can alter the synthesis and secretion of lipids and lipoproteins by liver cells, block human platelet aggregation and inhibit the synthesis of pro-aggregatory and proinflammtory eicosanoids by platelets and neutrophils[1-5]. Moreover, some reports have shown that resveratrol is a potential chemopreventive agent of cancer and it can prevent tumor growth and metastasis in human lung carcinoma, pancreatic cancer, prostate cancer, bronchial epithelioma cancer and breast cancer models[6-10]. The present investigation evaluated the potency of resveratrol on tumour cell growth and proliferation and on expression of cell cycle proteins in a transplantable murine hepatoma 22 model.

MATERIALS AND METHODS

Materials

Resveratrol was purchased from Sigma Co (USA). The resveratrol was dissolved and sterilized in dimethyl sulfoxide (DMSO) first and then diluted to the required working concentration in RPMI-1640 (Gibco, USA) containing 10 % calf serum (SiJiQing Co, Hangzhou, China). MS-110cdk1/p34cdc2 Ab-1 0.1 ml, MS-210 cyclin D1/bcl-1 Ab-1 0.1 ml, MS-338 cyclin B1 Ab-1 0.1 ml were purchased from Santa Cruz Co (USA). S-P ultra sensitive immunostaining kit was purchased from Maixin Biotechnology Developing Co (Fuzhou, China). Mouse hepatocellular carcinoma cell line H22 was kindly supplied by Cheng Wei (Center of Molecular Biology, First Affiliated Hospital, Xi' an Jiaotong University). Balb/c mice were purchased from the Animal Center of Xi' an Jiaotong University.

Methods

Suppressive effect of resveratrol on transplanted liver cancer of mouse H22 cells were first subcultured in RPMI 1640 containing 10 % fetal bovine serum. Then the cells were washed twice and resuspended in RPMI 1640 culture medium (1×10^8/ml). About 0.2 ml cell solution (including 2×10^7 cells) was taken and injected into the right groin of 5 Balb/c mice. After 14 days, when tumors of 3-5 mm in diameter formed in the right groin of these mice, they were taken out and sheared into small pieces of 1 mm³ under sterile condition. Forty Balb/c mice were anesthetized by using coelio-injection of pentobarbitone (70 mg/Kg) and laparotomy was performed. Under sterile condition their middle lobes of liver were punctured to form a 3 mm-long sinus tract and a small piece of tumor tissue was put into each sinus tract. Then these mice were divided into 4 groups randomly: one control group and 3 experimental groups. The experimental groups were designed to be injected with resveratrol (dissolved in DMSO and diluted to the working concentration of 25 mM in RPMI-1640 containing 10 % calf serum) at 5, 10 or 15 mg/kg body weight while the control group was designed to be given the same volume of the solution as for experimental group but absent of resveratrol. Twenty-four hours following liver tumour transplantation, each mouse was injected corresponding dosage of resveratrol into its abdominal cavity once a day for 10 days. These mice were then sacrificed on the following day after the last injection. After the maximum diameter and transverse length of tumour were measured, the hepatocellular carcinoma tissues and adjacent noncancerous liver tissues were sampled and fixed in 10 % formalin.
Measurement of tumour volume The tumour volume was

calculated using formula V=1/2 (maximum diameter x transverse length^2). The suppressive rate of tumour growth was calculated as [(mean V of tumour in control group - mean V of tumour in experimental group)/mean V of tumour in control group]×100 %.

Determination of protein expression of cyclin D1, cyclin B1 and p34cdc2 in murine transplantable liver cancer tissue
The tumour tissues and adjacent noncancerous liver tissues taken from therapeutic group (resveratrol in 10 mg/kg) and control group were 10 % formalin-fixed, paraffin-embedded and cut into 4 μm thick sections for staining. PBS of 0.01 mol· L^{-1} was used to substitute for primary antibody for negative control while a breast cancerous tissue expressing cyclin D1 and a tonsil tissue expressing cyclin B1 and cdk1/p34cdc2 were used for positive controls. The working concentration of each antibody was cyclin B1 (1:100), cyclin D1 (1:40) and cdk1/p34cdc2 (1:70), respectively. The staining procedures used were as described in S-P immunostaining kit. The sections were examined twice on different days by the same pathologist and the distribution of positively stained cells for each protein was evaluated semi-quantitatively by calculating the percentage of positive cells in nonoverlaping microscopic fields. The protein expression was then scored arbitrarily as "-" <5 % positive cells, "+" 5-50 % positive cells, "++" >50 % positive cells. χ^2 test was used to evaluate the statistical significance of the difference between experimental and control groups.

RESULTS

Suppressive effect of resveratrol on murine transplantable liver cancer
Except 2 Balb/c mice (one in control group and one in 15 mg/kg resveratrol therapeutic group), all the mice inoculated with hepatocarcinoma cell line H22 were successively transplanted with liver cancer. After treatment of the tumour bearing mice with 5, 10 or 15 mg/kg resveratrol for 10 days, the tumour size was reduced from 134 mm^3±40 mm^3 in control group to 105 mm^3±14mm^3, 85 mm^3±22mm^3 and 68 mm^3±17mm^3 in experimental groups, resulting in an inhibition rate of tumour growth of 21.6 %, 36.3 % and 49.3 %, respectively. The inhibition effect in the latter 2 therapeutic groups was significant compared with that in control group (all $P<0.05$) (Table 1).

Table 1 The suppressing effect of resveratrol on murine transplanted liver tissue

	Dose (mg/kg)	No. of mice (begin/end)	Tumour size (mm^3, $\overline{x}±s$)	GIR (%)
Control group	0	10/9	134±40	
Resveratrol	5.0	10/10	105±14[a]	21.6
	10.0	10/10	85±22[b]	36.3
	15.0	10/9	68±17[b]	49.3

[a]$P>0.05$, [b]$P<0.01$, vs control.

Effect of resveratrol on expression of cyclin D1, cyclin B1 and p34cdc2 in murine liver cancer tissue
Cyclin D1 immunoreactivity was observed in the nucleolus of tumour cell, cyclin B1 immunoreactivity and p34cdc2 immunoreactivity were observed in the cytoplasm of tumour cell. The expression level of cyclin D1, cyclin B1 and p34cdc2 in transplanted liver cancer tissue was significantly higher than that of adjacent noncancerous liver tissues in control group (All $P<0.05$) (Tables 2-4). Following treatment with resveratrol, a depressed expression of cyclin B (77.8 % vs 30.0 %, $P<0.05$) and p34cdc2 protein (88.9 % vs 40.0 %, $P<0.05$) was observed,

but the expression of cyclin D1 protein (66.7 % vs 60.0 %, $P>0.05$) did not change.

Table 2 Effect of resveratrol on the expression of cyclin D1 in murine liver tumor and adjacent noncancerous liver tissues

	No. of mice	Cyclin D1			Positive rate (%) (+, ++)
		-	+	++	
Noncancerous tissues	9	9	0	0	0
Control group	9	3	2	4	66.7[a]
Therapeutic group	10	4	3	3	60.0[b]

[a]$P<0.01$, vs noncancerous tissues; [b]$P>0.05$, vs control.

Table 3 Effect of resveratrol on the expression of cyclin B1 in murine liver tumor and adjacent noncancerous liver tissues

	No. of mice	CyclinB1			Positive rate (%) (+,++)
		-	+	++	
Noncancerous tissues	9	7	1	1	22.2
Control group	9	2	2	5	77.8[a]
Therapeutic group	10	7	2	1	30.0[b]

[a]$P<0.05$, vs noncancerous tissues; [b]$P<0.05$, vs control.

Table 4 Effect of resveratrol on the expression of p34cdc2 in murine liver tumor and adjacent noncancerous liver tissues

	No. of mice	p34cdc2			Positive rate (%) (+,++)
		-	+	++	
Noncancerous tissues	9	7	2	0	22.2
Control group	9	1	3	5	88.9[a]
Therapeutic group	10	6	3	1	40.0[b]

[a]$P<0.01$, vs noncancerous tissues; [b]$P<0.05$, vs control.

DISCUSSION

In 1997 Park et al, showed that resveratrol inhibited tumorigenesis in a mouse skin cancer model[11]. Since then, many studies have shown that resveratrol has suppressive effects on tumour cells in human lung carcinoma, prostate cancer, bronchial epithelioma and breast cancer models[12-14]. The ability of resveratrol to inhibit cellular events associated with tumour initiation, promotion, and progression might be attributed to its anticyclooxygenase activity (COX-1), inducing apoptosis of tumour cells, antagonizing mutation, antioxidation and anti-free radicals activity and its effect on cell cycle[15,16].

Recent studies have shown that many drugs exhibited their anti-tumour activity by interfering cell cycles. The cell cycle regulators have thus become a new target in drug discovery research. Of the 3 cell cycle regulators of cyclins, CDKs, and CKIs which have been found, CDKs is the core of the modulation network, which is positively regulated by cyclins and negatively by CKIs. Cyclin D1, a member of cyclins family, modulates mainly G1/S stage transition. Under normal conditions, the amount of cyclin D1 is invariable in G1 stage and its overexpression will lead to cells passing through G1/S without control, which is considered to be associated with carcinogenesis. Cyclin B1 is related mainly to the completion of M stage. CDC2 (CDK1) gene, a modulating core of G2/M inspect point, codes for p34cdc2 protein. The p34cdc2 combines with cyclin B1 to form MPF which plays an important role in the process from G2 stage to M stage. Studies have shown that cyclin B1-CDC2 was in activated state in many tumour cell lines no matter what the state of DNA was. Even

though cells had DNA damage, they could still enter the cleavage stage. Therefore, p34cdc2 and cyclinB1 were also associated with carcinogenesis[17].

In the present investigation, resveratrol was administered into murine abdomen and its potency on growth and proliferation of H22-innoculated tumour was evaluated by measuring the size of hepatoma and examining the expression of cell cycle proteins. The tumour size was found to be reduced by each dosage of 5, 10 or 15 mg/kg of resveratrol for 10 days. When the higher dosage of resveratrol was applied, the tumour size was significantly reduced, the inhibition rate of tumour growth by 10 or 15 mg/kg reached to 36.3 % and 49.3 %, respectively ($P<0.01$). We also found the protein expression of cyclin D1, cyclin B1 and p34cdc2 in murine transplanted liver cancer tissue was significantly higher than that in adjacent noncancerous liver tissues, suggesting that the overexpression of these cell cycle proteins may play a role in the onset and development of carcinoma. Compared with control group, a depressed expression of cyclin B1 and p34cdc2 protein was observed in the transplantable murine hepatoma following treatment with resveratrol, suggesting that the anti-tumour mechanism of resveratrol may be to prevent mitosis of tumor cells by suppressing the protein expression of cyclin B1and p34cdc2, and thus, interfering with the process of tumor cells from S stage to G2/M stage.

In short, the data presented in this paper suggest that resveratrol has an apparent antitumour activity, and blocking of S stage of tumour cells may be the underlying mechanism. The demonstration of modulating effect of resveratrol on cell cycle should provide certain theoretical basis for further study of resveratrol and other cell cycle interfering agents.

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