

Resveratrol Inhibits MMP-2 Expression of Hepatoma in Nude Mice

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Abstract: Resveratrol (trans-3, 4', 5-trihydroxystilbene) is a natural phytoalexin found in grapes and other plants that has anti-cancer and anti-inflammatory effects. The aim of this study was to find that resveratrol suppresses matrix metalloproteinase-2(MMP-2) expression in hepatoma *in vivo*. Tumor volume and invasion ability of liver transplanted tumor in nude mice were observed. MMP-2 mRNA and protein of the tumor were investigated by RT-PCR and Western-blot. NF-kappa B activity was measured by EMSA. Growth of the transplanted tumor was obviously by resveratrol at 50 mg kg⁻¹ (p<0.05) and 100 mg kg⁻¹ (p<0.05). Liver metastasis of the tumor was inhibited by 100 mg kg⁻¹ resveratrol (p<0.05). MMP-2 protein was down regulated due to decreased MMP-2 mRNA expression. NF-kappa B activity was also extensively inhibited by resveratrol at 50 and 100 mg kg⁻¹. The results suggest that resveratrol may inhibit growth and invasion ability by down regulate MMP-2 expression of hepatoma in nude mice. This effect was regulated by NF-kappa B pathway.

Key words: Resveratrol, invasion, hepatocellular carcinoma, matrix metalloproteinase-2, NF-kappa B, protein

INTRODUCTION

Primary liver cancer which consists predominantly of Hepatocellular Carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. The common treatment of liver cancer is suboptimal and the prognosis of patients is poor (El-Serag and Rudolph, 2007). Invasion and metastasis is one of main reasons of the poor prognosis. Several studies have reported a positive correlation between MMP-2 and invasion of malignant tumors (Hao *et al.*, 2007; Kawata *et al.*, 2007; Sillanpaa *et al.*, 2007).

Resveratrol (3, 5, 4-trihydroxystilbene), a polyphenol found in red wine that is associated with many health benefits, most notably the mitigation of age-related diseases, including neurodegeneration, carcinogenesis and atherosclerosis (Ferguson, 2001; Middleton *et al.*, 2000; Jang *et al.*, 1997). As a chemopreventive agent, resveratrol has been reported to interfere with some cellular events associated with initiation, promotion and progression of multi-stage carcinogenesis (Jang *et al.*, 1997). Although, the chemopreventive function on hepatic carcinoma of resveratrol has been well appreciated, the mechanisms by which resveratrol exerts its MMPs inhibition effects remain largely unknown. In the previous studies, resveratrol had effect on MMP-2 down regulation *in vitro*. The present study has been designed to find that resveratrol suppresses MMP-2 expression in hepatoma *in vivo*.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase and Taq DNA polymerase were from Invitrogen. Resveratrol were from Sigma (St Louis, MO, USA). Mouse monoclonal anti-human MMP-2 antibody was from NeoMarker (Fremont, CA, USA).

Cell culture: The hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC) and grown in DMEM (Invitrogen-Gibco, CA) with high-glucose (4.5 g L⁻¹) supplemented with 10% Fetal Calf Serum (FCS), 100 units mL⁻¹ penicilin and 100 mg mL⁻¹ streptomycin and sodium bicarbonate (2.0 g L⁻¹) at 37°C in 5% CO₂.

Animal treatment: BALB/c (nu/nu) nude mice (6 weeks of age) were supplied from the Experimental Animal Center of the Fourth Military Medical University. The animals were housed in climate-controlled quarters (24±1°C at 50% humidity) with a 12 h light/12 h dark cycle. HepG2 cells were harvested from tissue culture flasks with trypsin treatment. The cells were then washed with serum-free medium and suspended at a concentration of 5×10⁷ mL⁻¹ in serum-free medium. About 0.2 mL suspension containing 10⁷ cells was injected subcutaneously into the right flank of nude mouse. To evaluate the effect of resveratrol on tumor growth and liver metastasis, treatment of the drugs was carried out by subcutaneous

administration. For subcutaneous administration of the drugs, experimental animals (n = 10 for each group) were treated with 50 and 100 µg kg⁻¹ resveratrol three times/week beginning on the day of tumor cell implantation. Control mice were treated with normal saline (0.9%). Tumor volume was determined by measuring tumor size with calipers as resveratrol administration. All of the mice were killed 21 days after tumor implantation.

Protein extraction and Western blotting assay: Animals were killed by cervical dislocation at indicated times. For isolation of protein from mouse transplanted tumor, the transplanted tumor was excised, the fat was removed on ice and the tumor was immediately placed in liquid nitrogen and pulverized with a mortar. The pulverized tumor tissue was homogenized on ice for 20 sec with RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Tritonx-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 µg mL⁻¹ aprotinin, 10 µg mL⁻¹ leupeptinin] and cocktail proteinase inhibitor. Cell lysates were incubated on ice for 30 min and then spun down at 15000 rpm for 15 min at 4°C. The protein concentrations were quantitated by BCA protein assay kit (Pierce, IL). Equal amounts of 50 µg proteins were loaded onto a 10% polyacrylamide-SDS gel. After electrophoreses, the protein were transferred onto a nitrocellulose membrane (Milipore, MA). Membrane were blocked and probed with mouse anti human monoclonal MMP-2 antibody (Neomarker, USA) in 5% nonfat dried milk with a solution of TBS containing 0.05% Tween-20 overnight. After 30 min washes in TBS-0.05% Tween-20, blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 60 min followed by 30 min washes in TBS-0.05 Tween-20. Immunoreactive bands were detected using the enhanced chemiluminescence system (Pierce, IL) after exposure of the membrane to film for 30-60 sec.

Electrophoresis Mobility Shift Assay (EMSA): The nuclear extract from mouse transplanted tumor was prepared as described previously (Ferguson, 2001). EMSA was performed using a DNA-protein binding detection kit (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's protocol. Briefly, the oligonucleotide for NF-κB (5'- AGT TGA GGG GAC TTT CCC AGG C-3, 5'-TCA ACT CCC CTG AAA GGG TCC G-3') was labeled with [³²P] ATP by T4 polynucleotide

kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in a total volume of 25 µL containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, 0.1 mg mL⁻¹ sonicated salmon sperm DNA, 10 µg of nuclear extracts and 100,000 cpm of the labeled probe. After 50-min incubation at room temperature, 2 µL of 0.1% bromophenol blue was added and samples were electrophoresed through a 6% nondenaturing polyacrylamide gel at 150 V for 2 h. Finally, the gel was dried and exposed to an X-ray film.

Statistical analysis: Results were presented as mean±SD. Statistical significance was analyzed by one-way ANOVA test. The value of p<0.05 is considered significant.

RESULTS

Inhibitory effect of resveratrol on tumor growth in nude mice: To investigate the effect of resveratrol on tumor growth *in vivo*, HepG2 cells were injected subcutaneously into the right flank of nude mice (6 weeks old). Experimental animals (n = 10 each group) were treated with resveratrol (50 and 100 mg kg⁻¹) every 2 days beginning on the day of tumor formation. As shown in Table 1, tumor size showed a significant reduction in the 50 mg kg⁻¹ resveratrol treated group (18.48% inhibition) compared with the normal saline-treated group. A 42.48% inhibition of tumor growth in the 100 mg kg⁻¹ resveratrol treated group was observed compared with the normal saline-treated group. However, body weight in the resveratrol-treated groups was not reduced significantly compared with the normal saline-treated group.

Inhibitory effect of resveratrol on liver metastasis: Liver metastasis was observed in 8 mice from the control group, whereas 5 mice showed liver metastasis in the 50 mg kg⁻¹ group and 1 in the 100 mg kg⁻¹ group. A suppressive effect of the subcutaneous administration of 100 mg kg⁻¹ resveratrol on liver metastasis was significantly demonstrated when compared with the control group (p = 0.003) but 50 mg kg⁻¹ has no significant difference (p = 0.175). Subcutaneous administrations of 100 mg kg⁻¹ resveratrol clearly decreased the number of metastasis in the liver (Table 2).

Table 1: Effect of resveratrol on growth of HepG2 tumor xenografts in nude mice

Treatments	n	Inhibition of tumor growth		
		Tumor volume (mm ³) (%)	Inhibition ratio (%)	Body weight (g)
Normal saline (0.9%)	10	303.64±13.07	-	19.35±0.35
50 mg kg ⁻¹ Res	10	247.54±11.80*	18.48	17.70±1.13
100 mg kg ⁻¹ Res	10	174.66±12.73*	42.48	21.70±0.99

*p<0.05

Table 2: Inhibitory effect of resveratrol on liver metastasis

Treatment	Inhibition of liver metastasis	
	Number of mice	Metastasis ratio (%)
Normal saline (0.9%)	8	80
50 mg kg ⁻¹ Res	5	50*
100 mg kg ⁻¹ Res	1	10#

*p>0.05 vs control; #p<0.05 vs control

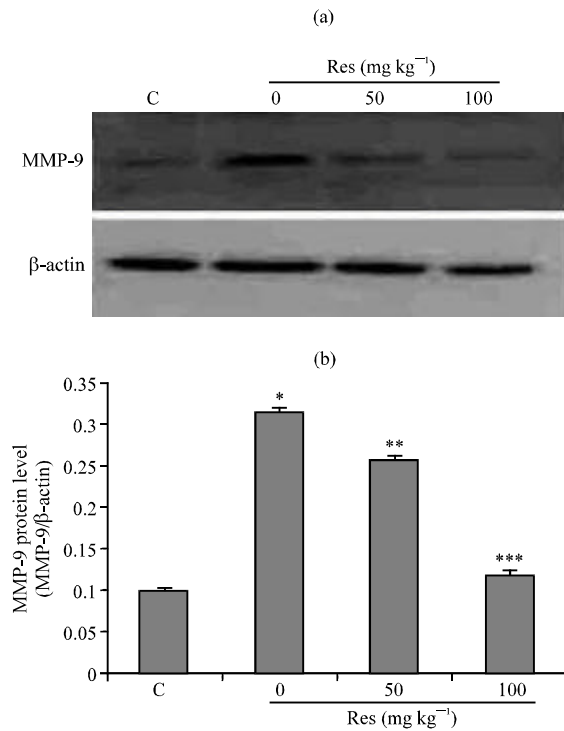


Fig. 1: Resveratrol inhibits MMP-2 protein expression of liver transplanted tumor in nude mice. Western-blot showed that MMP-2 protein was successfully inhibited in liver transplanted tumor of nude mice. The expressions were representative of three independent experiments, showing similar trends. *p<0.05 vs control, **p<0.05 vs Res 50 mg kg⁻¹ group

Resveratrol inhibited MMP-2 protein production of transplanted liver tumor in nude mice: To determine whether the above inhibition of tumor invasion is related to direct inhibition of MMP-2 enzyme activity or related to inhibition of MMP-2 protein expression, Western blot assay of MMP-2 protein in transplanted liver tumor of nude mice was carried out. Figure 1 showed that MMP-2 protein was increased in the tumor. Treatment with resveratrol caused a decrease in MMP-2 protein production. MMP-2 protein was suppressed by resveratrol at 50 and 100 mg kg⁻¹ in a concentration dependent manner (Fig. 1).

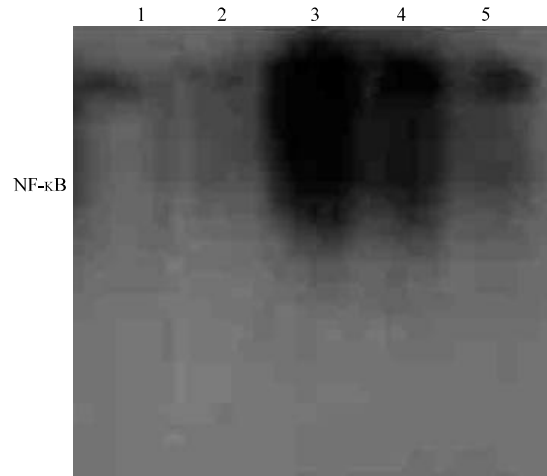


Fig. 2: Effect of resveratrol on the activation of NF- κ B of transplanted liver tumor of nude mice. Nude mice were treated topically with resveratrol at a dose of 0, 50 or 100 mg kg⁻¹ dissolved in 0.2 mL acetone. The epidermal nuclear extracts were prepared and incubated with the radiolabelled oligonucleotides containing NF- κ B consensus sequence for analysis by EMSA. Lane 1, free probe alone (no nuclear extracts); Lane 2, Acetone control; Lane 3, resveratrol 0 mg kg⁻¹; Lane 4, resveratrol 50 mg kg⁻¹; Lane 5, resveratrol 100 mg kg⁻¹

Resveratrol and dexamethasone inhibited NF- κ B activation of transplanted liver tumor in nude mice: To determine whether MMP-2 down regulation related to activation of transcriptional factors NF- κ B, EMSA assay was also performed using consensus oligonucleotides for NF- κ B. As shown in Fig. 2, tumor had a substantial increase in NF- κ B activation which could be inhibited by 50 and 100 mg kg⁻¹ resveratrol.

DISCUSSION

Invasion and metastasis is fundamental property of malignant cancer and the main factors related to the poor prognosis. Tumor invasion and metastasis are a multi-stepped and complex process that includes cell division and proliferation, proteolytic digestion of the ECM, cell migration through the basement membranes to reach the circulation system and the remigration and growth of tumors at metastatic sites (Hidalgo and Eckhardt, 2001). MMPs play a major role in promoting angiogenesis and tumor metastasis (Kostoulas *et al.*, 1999). An enhanced expression of MMP-2 has been shown to be associated with the progression and invasion of tumors (Liu *et al.*, 2010a; Stankovic *et al.*, 2010;

Hung *et al.*, 2010). The red wine polyphenol resveratrol has been recognized as a potent chemopreventive agent in several laboratory studies. The inhibition effect of resveratrol has been attributed to its ability to suppress matrix metalloproteinase-2 expression in different tumor (Yang *et al.*, 2009; Gagliano *et al.*, 2005; Weng *et al.*, 2010; Liu *et al.*, 2010b). In spite of an extensive investigation of matrix metalloproteinase-2 suppression of resveratrol *in vitro*, few studies have focused on resveratrol inhibiting MMP-2 expression of hepatic carcinoma *in vivo*.

MMP-2 has been investigated over expression in hepatocellular carcinoma cell line in the previous investigation. As shown in the study MMP-2 expressed more in transplanted tumor than in sid-tumor in nude mice. In order to investigate suppression ability of resveratrol on MMP-2, different concentration of resveratrol was subcutaneous injected. In the present study, we found that topical application of resveratrol at 50 mg kg⁻¹ and 100 mg kg⁻¹ resulted in a dose-related decrease in the levels of MMP-2 expression by inhibiting MMP-2 gene transcription. Resveratrol was found recently to have obviously direct inhibitory effect on MMP-2 expression, so inhibition of resveratrol on MMP-2 gene expression may be one of its anticancer mechanisms.

In regard to the mechanism by which resveratrol modulates gene expression, resveratrol was found to suppress PMA-mediated activation of NF-kappa B in nude mice. MMP-2 promoter contains cis-acting regulatory elements and transcription factors, including AP-1 and NF-kappa B which participate in the regulation of the MMP-2 gene (Fong *et al.*, 2010). Resveratrol has been suggested to activate NF-kappa B as a molecular mechanism of MMP-2 expression in human cultured glioblastoma cells (Gagliano *et al.*, 2005). The nuclear fractions from transplanted hepatoma of nude mice with or without resveratrol pretreatment were analyzed by the electrophoretic mobility gel shift assay. Resveratrol treatment attenuated activation of NF-kappa B in transplanted hepatoma of nude mice.

Besides NF-kappa B, the expression of MMP-2 is also regulated by another transcription factor nuclear AP-1 (Shieh *et al.*, 2010). Results from both cultured cell line and animal model experiments have suggested that resveratrol can inhibit tumor promoter-induced activation of AP-1 (Kutuk *et al.*, 2006). Since resveratrol has been shown to depress MMP-2 expression in hepatoma of nude mice via inhibiting NF-kappa B activity, the possibility of AP-1 as a molecular target of resveratrol is currently underway.

CONCLUSION

In conclusion, it has shown that resveratrol could inhibit MMP-2 expression of liver tumor in nude mice.

This inhibition may be mediated by the regulation of NF-kappa B. Future study will investigate the effects of resveratrol in control of hepatocellular carcinoma cell in clinics.

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