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Effect of As_2O_3 and Ginsenoside Rg3 on Inhibiting Angiogenesis in Rabbit Liver VX2 Tumor

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Abstract

Liver cancer is an aggressive disease with a poor outcome. Here, we implicate the roles of arsenic trioxide (As_2O_3) and Ginsenoside Rg3 (GRg3) in VX2 carcinoma model of rabbits through CD31 and vascular endothelial growth factor (VEGF). Rabbits VX2 liver cancer models were established, then 24 rabbits were randomly divided into 3 groups with the same number (As_2O_3, GRg3 and control). Liver function and the levels of VEGF and CD31 were assessed. The tumor weight and levels of VEGF and CD31 in As_2O_3 and GRg3-treated rabbits were lower than those in controls, while no significant differences between As_2O_3 and GRg3 groups. There was no significant difference in the liver function among the groups. In conclusion, our findings implicate decreases in the tumor weight and the levels of VEGF and CD31 in the rabbits VX2 liver cancer induced by As_2O_3 and GRg3, while no significant differences between As_2O_3 and GRg3. GRg3 might be much more convenient than As_2O_3 in clinical application.

Keywords: Arsenic trioxide; Ginsenoside Rg3; Vascular endothelial growth factor; CD31; Hepatic carcinoma

Introduction

Liver cancer is an aggressive disease with a poor outcome. Nowadays, increasing attention has been paid to the treatment of neoplasm angiogenesis [1], besides traditional surgery, radiotherapy, and chemotherapy, especially the use of traditional Chinese medicine. Arsenic trioxide (As_2O_3) and GRg3 are the main component of Chinese medicine White arsenic and ginseng, respectively. As_2O_3 and GRg3 play an important role in tumor angiogenesis inhibition. However, few studies have explored the differences between As_2O_3 and GRg3 on liver cancer [2].

There was a positive correlation between microvessel density and tumor recurrence or survival. Meanwhile, various immunohistochemical markers were used to estimate microvessel proliferation in tumors, such as pan-endothelial markers CD31 [3] and antibodies against angiogenic proteins like vascular endothelial growth factor (VEGF) [4]. In this study, we investigated the roles of As_2O_3 and GRg3 in VX2 carcinoma model of rabbits through CD31 and VEGF.

Materials and Methods

Animals

Twenty-four New Zealand white rabbits of 16 weeks 2.0~2.2 kg, male: female=1:1, animal license keySCXK Liao 2003-0013were obtained from the Center for Experimental Animals at China Medical University. All experiments and surgical procedures were approved by the Institutional Animal Care and Use Committee, which complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize animal suffering. With the guidance of ultrasound, VX2 carcinoma masses were injected into the rabbits' livers using percutaneous puncture-inoculation method [5]. Then Rabbits VX2 cancer model were identified by CT enhancement contrast successfully. After 6 weeks, the rabbits were randomly divided into 3 groups with the same number (n=8, male: female=1:1, As_2O_3 group, GRg3 group and control group). As_2O_3 treated group: Referring to the instruction of arsenous acid (Harbin Yida pharmaceutical Co., Ltd., China), getting the equivalent dose for rabbit (0.6 mg/kg), which is calculated according to the ratio of body surface area of rabbit to human. The equivalent dose was diluted to 3 ml and injected via marginal ear vein slowly with 1 ml/day. GRg3 treated group: Referring to the instruction of Shenyi capsule (Jilin Yatai pharmaceutical Co., Ltd., China), getting the equivalent dose for rabbit (10 mg/kg), which is calculated according to the ratio between rabbit's and human's body surface area. The drug was dissolved by normal sodium, giving 2 ml per rabbit via intragastric administration. Control group: The animals were given NS 2 ml per rabbit via intragastric administration. All the animals were sacrificed after given drug for continuous 7 days. Liver function was detected by the levels of alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), and total bilirubin (TBIL, mmol/L) using a biochemical autoanalyzer (Model LX 20; Beckman, CA, USA).

The pathological features observation

The whole neoplasm tissue of each rabbit was collected and weighed. For pathological studies, all histopathological tests were performed using standard laboratory procedures. The excised tissues were fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned into 3 μm thick sections using a sliding microtome.

The additional sections of tumors were stained by immunohistochemical method for VEGF and CD31. The slices were deparaffinized in xylene and rehydrated with gradual ethanol to water. To expose antigen epitopes, the slices were heated in a sodium citrate buffer (pH 6.0) in a microwave at a power of 750W for 3.5 minutes. Treated slices were stored at room temperature for 30 minutes and washed in phosphate-buffered saline (PBS, pH 7.4). After

preincubation in methanol with 3% H_2O_2 for 20 minutes, slices were blocked by serum-free blocking solution at room temperature for 10 minutes, and incubated with primary monoclonal antibody of CD31 (Wuhan Boster Biological Technology Co., Ltd., China) and VEGF (Wuhan Boster Biological Technology Co., Ltd., China), respectively. After washing in PBS, the slices were incubated with biotin-conjugated second antibody and three washes of PBS. Then, sections were incubated for 30 min in streptavidin–peroxidase and washed three times in PBS. Then, sections were reacted with a solution of 0.05% of 3, 30-diaminobenzidine (DAB, Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., China), 0.01% H2O2 in 0.1 M PBS. Incubation times varied from 3 to 10 min depending upon the expressing levels of DAB reaction product monitored with a microscope. Finally, they were subjected to 3, 30-diaminobenzidine and counterstained by hematoxylin for 3 min, dehydrated, rinsed, and mounted with glycerin gelatin for histological examination.

Judgment standard of VEGF

VEGF protein is distributed in cytoplasm. The sections were microscopically analyzed at X40 (objective × 4 and ocular ×10) magnification for the tumor tissues. Then images were obtained at a magnification of ×400 (objective × 4 and ocular × 10) to analyze the VEGF protein-positive cells (stained brown). The immunostaining was evaluated by using a semi quantitative scale: (-) ≤ 4.99% of VEGF-positive tumor cells, (+)5.00%15.99% of VEGF-positive tumor cells, (++)16.00% 50.99 of VEGF-positive tumor cells, and (+++) ≥ 51.00% of VEGF-positive tumor cells.

Judgment standard of microvessel density (MVD)

Microvessel density was assessed by immunostaining of CD31. Most vascular areas (so called hot-spots) in the tumor were located at low magnification (X40) and then counted at X400 magnification. Each positive endothelial cell or cluster of cells in contact with a spot was counted as one single microvessel (stained brown). The mean vessel count from 5 different fields was used as CD31-MVD.

Statistical analysis

All the statistical analyses were performed using SPSS software (Version 12.0; SPSS Inc, Chicago, IL, USA) with the analyzer blind to the treatment of each group. Data were expressed as mean ± standard deviation (SD) and significant level was set P=0.05. The comparisons of the tumor weight, MVD and liver function among the As_2O_3 group, GRg3 group, and control group were made using one-way ANOVA. When the F-value indicated significance, least-significant difference or Tamhane's T2 post hoc comparisons were made as appropriate to correct for multiple comparisons. The comparisons of the expression levels of VEGF among the groups were made using chi-square test with continuity correction Fisher's exact test. All P-values were two-tailed.

Results

Effects of As_2O_3 and GRg3 on tumor weight and liver function

There were significantly differences of the tumor weight among the three groups (P=0.021). The tumor weight of As_2O_3 group (5.9 ± 2.7 g, P=0.013) and GRg3 group (6.3 ± 3.8 g, P0.033) were significantly lower than that of the controls (11.7 ± 6.2)g. However, no significant difference was observed between As_2O_3 and GRg3 group (P>0.05). Thus, in our hands, the As_2O_3 and GRg3 decreased tumor growth.

In addition, the results showed that no significant difference in the levels of ALT (F2,21=1.81, P=0.19), AST(F2,21=0.80, P=0.46), and TBIL (F2,21=0.30, P=0.75) among the three groups.

As_2O_3 and GRg3 down-regulated CD31 and tumor angiogenesis

CD31, immunohistochemical marker, was used to estimate microvessel proliferation in the tumor [3]. In the present study, we investigated the levels of CD31 following the effect of As_2O_3 and GRg3 on the inhibiting angiogenesis in hepatic VX2 carcinoma. Our data showed that, the CD31 staining-positive vascular endothelial cells could be observed clearly on the single endothelial cell or on the endothelial distributing in clusters (Figures 1a, 1b, and 1c). Except small blood vessel and central vein in portal area, the protein of CD31 was seldom expressed in the normal liver sinus tissue. Furthermore, the CD31 staining was strongly expressed in the sinusoid blood vessel of tumor lesions.

The CD31-MVD of As_2O_3 group (Figure 1a, 18.38 ± 5.21, P=0.006) and GRg3 group (Figure 1b, 19.13 ± 5.57, P=0.003) were significantly lower than that of the controls (Figure 1c, 27.75 ± 5.12). Meanwhile, there was no significant difference of CD31-MVD between the groups of As_2O_3-treated and GRg3-treated (P=0.785).

Taken together, our results demonstrated that the As_2O_3-treated and GRg3-treated down-regulated tumor angiogenesis in rabbit VX2 carcinoma.

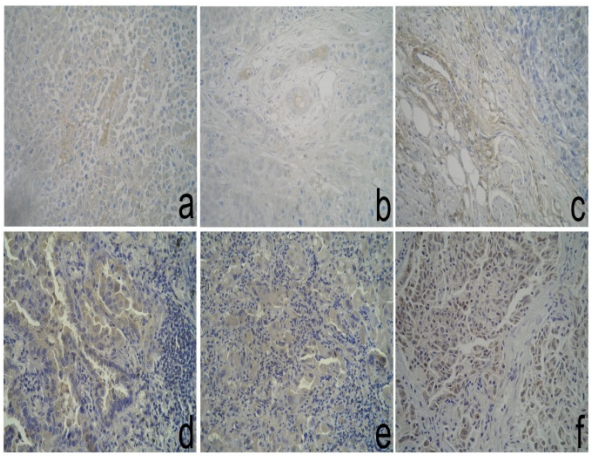

Figure 1: Immunohistochemical staining shows the decrease expression of CD31 and VEGF (n=8 rabbits for each group, original magnification X200); Representative immunohistochemical staining with CD31-MVD and VEGF monoclonal antibodies in VX2 liver tumors in each group; Microvessels are identified by brown; The levels of CD31-MVD in As2O3 group (a) and GRg3 group (b) were significantly lower than control group (c); Positive VEGF are recognizable as intensely stained in tumor cell cytoplasm; It is evident that the abundance of VEGF-positive tumor cells is lower in the As2O3 group (d) and GRg3 group (e) than in the control group (f).

As2O3 and GRg3 down-regulated VEGF

VEGF plays a key role in the formation and growth of normal blood vessels and in tumor angiogenesis [6]. In the present study, we investigated the levels of VEGF using immunohistochemical techniques.

The result showed that VEGF was expressed in all groups (Figures 1d, 1e, and 1f). Staining for VEGF was strongly detected in the control group. Furthermore, the intensity of staining and expression of VEGF in As2O3 ($\chi2$=7.356, P=0.024, Figure 1d) and GRg3-treated rabbits ($\chi2$=6.651, P=0.024, Figure 1e) were significantly lower than those of controls (Figure 1f). Meanwhile, there was no significant difference between the As_2O_3 and GRg3-treated groups ($\chi2$=0.602, P=1.000), Table 1.

Group	VEGF			
	-	+	++	+++
As2O3	0	4	3	1
GRg3	0	3	4	1
Control	0	0	2	6

Table 1: Comparisons of Tissue VEGF levels among the groups.

Discussion

The major findings of this study with rabbits exposed to hepatic VX2 carcinoma were that (1) Tumor mass weight was decreased compared to the controls, (2) liver function was similar among the groups, (3) CD31-MVD was downregulated compared to the controls, (4) the levels of VEGF was significantly lower than control levels. These findings demonstrate that As_2O_3 and GRg3 may be involved in the inhibition of angiogenesis in hepatic VX2 carcinoma model of rabbits.

Hepatic carcinoma was one of the most common and aggressive tumors. Even after careful surgical excision, radio and chemotherapy, hepatocellular carcinoma showed a poor outcome. It was generally accepted that angiogenesis was one of the most important factors in the progression of malignant tumors [7,8]. Thus, many efforts had focused on new therapy approaches based on the inhibition of angiogenesis for liver cancer [9].

Most of the malignant tumors developed their own vascular networks by secreting growth factors, such as VEGF, which stimulate endothelial migration and proliferation [10]. In the present study, VEGF levels decreased significantly in As_2O_3- and GRg3-treated rabbits relative to the controls. Thus, we speculated that it was the key point for the effect of As_2O_3 and GRg3 on liver cancer.

As_2O_3 has been used for refractory and recurrent acute promyelocytic leukemia and achieved a great success in China. Recently, more attention paid to the use of As_2O_3 on the therapy of hepatoma [5]. It has been reported that As_2O_3 prolonged the median survival time of the patients with middle and late hepatoma, especially for the patients treated for the first time. Furthermore, As_2O_3 stabilized the life quality and improved the clinical symptom, while there was no obvious toxic side effect. For advanced liver cancer, combination therapy of As_2O_3 and chemotherapy decreased the incidence of intrahepatic and extrahepatic metastasis of tumors compared to the chemotherapy used alone. However, the mechanism was still unclear. One of the reasons might be the cellular toxicity of As_2O_3, which induced the differentiation of tumor cell, promoted the apoptosis of tumor cell, and inhibited the proliferation of tumor cells and anti-angiogenesis.

GRg3 is a monadelphous element derived from ginseng. It has been proved that GRg3 inhibitted the growth of vascular endothelial cells, stopped the formation of vascular networks of tumors, decreased the intracellular concentration of $Ca2^+$, and inhibited $Ca2^+$ implantation on vascular wall and $Ca2^+$ infiltration to inner vascular wall both *in vivo* and *in vitro*. Moreover, GRg3 obviously inhibited the production and expression of VEGF and basic fibroblast growth factors which decreased the number of neovessel. Thus, it might be the reason that GRg3 inhibited the development of liver tumor growth.

To the best of our knowledge, few studies detected the differences between As_2O_3 and GRg3 in hepatic carcinoma. Presently, we showed that As_2O_3 and GRg3 downregulated tumor mass in hepatic VX2 carcinoma model of rabbits, involved in VEGF and CD31-MVD. Furthermore, no significant differences were observed among the groups in liver function. However, the exact mechanism has not been identified and need to be investigated with additional experiments. Notably, GRg3 was oral administration, while As_2O_3 was injection. Thus, our results suggested that GRg3 might be much more convenient than As_2O_3 in clinical application.

Conclusion

In conclusion, the levels of VEGF and CD31-MVD decreased both in As_2O_3 and GRg3-treated groups in liver VX2 tumor, and no change in liver function. GRg3 might be much more convenient than As_2O_3 in clinical application.

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