BRIEF ARTICLES

Effect of preoperative transcatheter arterial chemoembolization on angiogenesis of hepatocellular carcinoma cells

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Abstract

AIM: To evaluate the effects of four types of preoperative transcatheter arterial chemoembolization (TACE) on angiogenesis of hepatocellular carcinoma (HCC) cells.

METHODS: A total of 136 patients with HCC underwent liver resection. One to five courses of TACE prior to liver resection were performed in 79 patients (TACE group), in which one to four courses of chemotherapy alone were performed in 11 patients (group A); one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B); one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C); one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). The other 57 patients only received liver resection (non-TACE group). The microvessels were marked by CD31. The expression of CD31 and vascular endothelial growth factor (VEGF) protein were detected by immunohistochemical methods.

RESULTS: The mean microvessel density (MVD) in HCC cells was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein in HCC cells were significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). MVD and the expression of VEGF protein were positively correlated. Mean MVD and the expression of VEGF protein were closely related to the number of courses of TACE and the interval of TACE.

CONCLUSION: Four different types of preoperative TACE regimens enhanced angiogenesis in HCC cells by up-regulating the expression of VEGF protein. It is necessary to repress angiogenesis of liver cancer after TACE.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asian countries. It is responsible for more than 250 000 deaths worldwide each year, 40% of which occur in China ranking HCC second after gastric carcinoma[1-3]. Surgical resection is recognized as the most effective treatment method for patients with HCC[4]. Although recent advances in treatment have helped prolong the survival of patients with HCC, they consequently increase the risk of intrahepatic recurrence and extrahepatic metastasis. Only a minority of patients currently diagnosed with HCC may benefit from this radical option[5].

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with HCC[6-11]. Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. There have been few reports comparing the efficacy of different TACE regimens in patients with HCC[12-14].

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for solid tumor growth and metastasis[15]. Vascular endothelial growth factor (VEGF) is a strong angiogenesis factor in HCC[16], and plays an important role in the development and prognosis of liver cancer. In the present study, we examined the effects of the four main types of TACE used clinically (pure intra-arterial chemotherapy, chemotherapy plus lipiodol, chemotherapy plus lipiodol plus gelatin sponge, and chemotherapy plus lipiodol plus alcohol plus gelatin sponge) on angiogenesis of HCC cells *in vivo*.

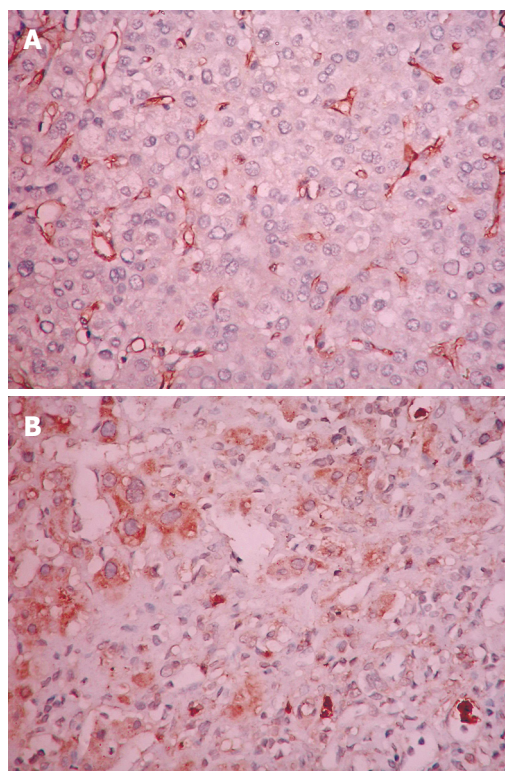## MATERIALS AND METHODS

Patients
From February 1992 to February 2001, a total of 136 patients with HCC were referred to our hospital for surgery, of which, 122 were men and 14 were women with a mean age of 45 years (range: 20 to 70 years). A diagnosis of HCC was obtained for all patients by preoperative ultrasound (US) and/or computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or plasma AFP levels, and was then confirmed by biopsy.

Surgical procedure
The patients were randomly divided into two groups. In the TACE group, 79 patients underwent 1-5 courses of chemoembolization prior to liver resection, in which one to four courses of chemotherapy alone were performed in 11 patients (group A), one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B), one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C), and one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). Fifty patients underwent one course of TACE, 19 patients underwent two courses of TACE and 10 patients underwent three or more courses of TACE during an interval of 52.8 ± 12.2 d (mean $\pm$ SD). Twenty-five patients had an interval of one month or less, 29 patients had an interval of two months or less, 16 patients had an interval of three months or less and 9 patients had an interval of more than three months. In the non-TACE group, 57 patients received initial liver resection without preoperative TACE. The extent of liver resection carried out was based on the location of the tumor, the severity of concomitant liver cirrhosis and preoperative liver reserve function.

Immunohistochemical methods
The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using anti-CD31 antibody and anti-VEGF antibody (LSAB Kit, Dako). Breast cancers were used as positive controls. Negative controls were generated by substituting the primary antibody with phosphate-buffered saline (PBS). CD31 and VEGF immunostained cells showed a brownish-yellow color in the cytoplasm (Figure 1A and B). Microvessel

Figure 1 Expression of CD31 (A) and VEGF (B) protein detected by immunohistochemical methods after chemotherapy combined with ethanol, iodized oil and gelatin sponge (Dako Envision, peroxidase method × 400).

density (MVD) counting followed the method of Weidner *et al*[17], i.e. first, the regions with the highest density of CD31-positive cells were chosen and counted under a low-power microscope ($\times$ 40). Then microvessel numbers were counted under a high-power microscope ($\times$ 400). Each isolated brown vascular endothelial cell or cluster of endothelial cells was counted as a vascularization. VEGF counting followed the method of Park *et al*[18], i.e. negative (-) was < 5% of positively stained cells, weakly positive (+) was 5% to 15% of positively stained cells, moderately positive (++) was 15% to 50% of positively stained cells and strongly positive (+++) was > 50% of positively stained cells. All slides were reviewed and scored in a blind test by two observers without knowledge of the corresponding clinical data. A few cases with discrepant scoring were jointly re-evaluated until agreement was reached.

Statistical analysis
MVD was expressed as mean $\pm$ SD and analyzed using the two-sample *t*-test for the two groups, and by analysis of variance for multiple comparisons. VEGF was analyzed using table χ^2 test. The correlation between MVD and VEGF was analyzed using Pearson correlation analysis. *P*-value < 0.05 was considered statistically significant.

RESULTS

Correlation between methods of TACE and expression of CD31 and VEGF protein
The mean MVD was (47.71 ± 23.33), (56.05 ± 22.45),

Table 1 Expression of VEGF protein in groups A, B, C, D, and the non-TACE group

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
A	11	2 (18.2)	4 (36.4)	3 (27.3)	2 (18.2)
B	33	5 (15.2)	6 (18.2)	9 (27.3)	13 (39.4)
C	23	2 (8.7)	6 (26.1)	4 (17.4)	11 (47.8)
D	12	1 (8.3)	2 (16.7)	4 (33.3)	5 (41.7)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 2 Correlation between expression of CD31 and VEGF protein

Group	Cases	MVD
VEGF (-)	27	35.47 ± 17.35
VEGF (+)	34	44.12 ± 15.84
VEGF (++)	33	52.56 ± 17.29
VEGF (+++)	42	60.72 ± 23.46

Table 3 Correlation between courses of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
One-course	50	5 (10.0)	14 (28.0)	12 (24.0)	19 (38.0)
Two-course	19	3 (15.8)	4 (21.1)	3 (15.8)	9 (47.4)
Three-, four- or five-course	10	2 (20.0)	0 (0.0)	5 (50.0)	3 (30.0)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 4 Correlation between interval of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
1 mo interval	27	4 (14.8)	7 (25.9)	7 (25.9)	9 (33.3)
1-2 mo interval	28	2 (7.1)	7 (25.0)	6 (21.4)	13 (46.4)
2-3 mo interval	16	3 (18.8)	2 (12.5)	5 (31.3)	6 (37.5)
> 3 mo interval	8	1 (12.5)	2 (25.0)	2 (25.0)	3 (37.5)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

(54.36 ± 24.46), (51.90 ± 19.41) and (44.36 ± 17.67) in groups A, B, C, D and the non-TACE group, respectively. The mean MVD was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was significantly higher in groups B, C and D than in group A or the non-TACE group ($\chi^2 = 12.63$, $P < 0.05$) (Table 1). When the expression of VEGF protein was increased, MVD increased significantly, and both were positively correlated (Pearson correlation, $r = 0.445$, $P < 0.05$) (Table 2).

Correlation between courses of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (54.01 ± 23.83), (53.38 ± 22.64) and (51.94 ± 22.64) in the non-TACE group, the one-course TACE group, the two-course TACE group and the three-, four- and five-course TACE group, respectively. The mean MVD was significantly higher in the one-course TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE groups than in the non-TACE group ($\chi^2 = 16.786$, $P > 0.05$) and decreased as the courses of TACE increased (Pearson correlation, $r = 0.331$) (Table 3).

Correlation between interval of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (49.20 ± 19.84), (55.30 ± 23.31), (61.48 ± 26.63) and (44.25 ± 17.52) in the non-TACE group, the 1 mo-interval TACE group, the 1-2 mo interval TACE group, the 2-3 mo interval TACE group, and the > 3 mo interval TACE group, respectively. A comparison between the groups showed that the mean MVD was higher in the 1 mo-interval TACE group, the 1-2 mo interval TACE group and the 2-3 mo interval TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE interval groups than in the non-

TACE group ($\chi^2 = 12.488$, $P = 0.407$) and was highest in the 1-2 mo interval TACE group (Table 4).

DISCUSSION

TACE is one of the most common and effective palliative treatments. The prognosis of patients treated with TACE depends not only on the use of an effective TACE regimen but also on tumor factors[12]. According to the literature, very limited data are currently available regarding the molecular mechanism of TACE treatment in patients with HCC[13-15]. We believe that the current study is the first to detail the correlations between the expression of CD31 and VEGF protein and different TACE regimens, courses of TACE and interval of TACE.

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for the growth and metastasis of solid tumors[15]. VEGF is a strong factor in the angiogenesis of HCC[16], and plays an important role in the development and prognosis of liver cancer. It also has a biological effect by combining its specific VEGF receptor (vascular endothelial growth factor receptor, VEGFR), of which VEGFR-1 and VEGFR-2 are mainly distributed in vascular endothelial cells. Binding of VEGFR-1 and VEGF allows vascular endothelial cell migration, maintains tubular structure and regulates vascular permeability. Binding of VEGFR-2 and VEGF also promotes vascular endothelial cell proliferation and maturation[19]. This study showed that MVD of HCC was positively correlated with the expression of VEGF protein.

Whether angiogenesis of liver cancer after TACE is enhanced is still controversial. Some reports have suggested that the mean MVD of HCC specimens before and after TACE were not statistically significantly

different[20-23]. However, there are other reports which show that the mean MVD was significantly higher in the TACE group than in the non-TACE group[24-26]. The current study showed that the mean MVD of HCC was significantly higher in the TACE groups than in the non-TACE group. The expression of VEGF protein was significantly higher in the TACE groups than in the non-TACE group. After chemoembolization, the expression of VEGF protein increased, and the change in expression and MVD showed a significant positive correlation. It has also been reported that serum VEGF in patients with liver cancers was significantly increased after TACE[27,28]. Tumor tissue ischemia and hypoxia after TACE are important in promoting increased VEGF expression[29]. Therefore, it is necessary to repress angiogenesis in liver cancer after TACE.

The study on the effects of the interval between surgical resection of the tumor and the end of embolization on MVD, revealed that MVD was not significantly different in the group with an interval of less than 30 d or in the group with an interval of more than 90 d, but was significantly increased in the group with an interval of 31-90 d compared with the control group. This showed that after chemoembolization, tumor hypoxia-ischemia results in a series of biochemical changes which cause increased angiogenesis and a gradual increase in MVD, reaching a peak in 1-3 mo. More than three months later, the blood supply to the residual tumor improves, the formation of tumor angiogenesis slows, the residual cancer cells grow, infiltration occurs and damage to generated tumor angiogenesis occurs.

The mean MVD and expression of VEGF protein in liver cancer has a tendency to decrease as the time from TACE therapy increases, although this difference was not significant. It was reported that survival after a number of pre-operative TACE treatments was significantly better than that after a single TACE treatment[30]. Therefore, multiple pre-operative TACEs should be carried out in suitable patients.

In conclusion, the present study demonstrated that angiogenesis of residual HCC cells following treatment with four types of TACE is significantly increased and is positively correlated with the expression of VEGF protein. The effect of TACE on angiogenesis of HCC cells has a close correlation with the number of courses of TACE and the interval of TACE.

COMMENTS

Background
Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with hepatocellular carcinoma (HCC). Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. However, there have been few reports comparing the effects of different TACE regimens on angiogenesis of HCC cells.

Applications
According to the results of this study, it is necessary to repress angiogenesis of liver cancer after TACE.

Terminology
TACE indicates transcatheter arterial chemoembolization; MVD indicates microvessel density.

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TACE stimulates angiogenesis. It would be very useful from a clinical standpoint to identify which type of TACE stimulates more angiogenesis and give appropriate "prophylactic" anti-angiogenic therapy. The authors have the merit of confirming that TACE is an angiogenesis stimulating procedure and also give new information on this process -- e.g. angiogenesis peaks about 1 mo after TACE and then seems to wane off. The number of observations and the histological specimen (surgical) are adequate.

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