

Dynamic Expression and Alteration of Vascular Endothelial Growth Factor and Angiopoietin in Hepatocyte Malignant Transformation and Hepatocarcinogenesis

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(Received: 03 March 2013; accepted: 14 April 2013)

To investigate the dynamic expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2), and antiangiogenic therapy on hepatocyte malignant transformation. Hepatoma models were induced with 2-fluorenylacetylamide (2-FAA) on male Sprague-Dawley rats, thalidomide was administered intragastrically (100 mg/kg body weight) to intervene HCC progress. VEGF expression was analyzed by immunohistochemistry, and its level was quantitatively detected by enzyme linked immunosorbent assay or by Western blotting. An increasing tendency of VEGF or Ang expressions in the 2-FAA group was found from hepatocyte denatured, precancerous, and cancerous stages confirmed by HE staining with highest values in the HCC group than those in controls ($P < 0.001$). The positive relationship was closely found between VEGF and Ang level ($P < 0.01$) with abnormality at the early stage. Interestingly, the morphologic changes of hepatocytes in the thalidomide group generated only punctiform denaturation or slightly necrosis at the early or middle stages, and nodular hyperplasia or a little atypical hyperplasia at the final stages, with the down-regulation of VEGF ($\div^2 = 8.024$, $P < 0.001$) and Ang ($\div^2 = 9.93$, $P < 0.001$) comparative with those in the 2-FAA group. The over-expressions of VEGF and Ang-2 are associated with hepatocyte malignant transformation, and antiangiogenic treatment can down-regulate the both expression and delay hepatoma formation.

Key words: Hepatocellular carcinoma, Rat hepatoma model, Vascular endothelial growth factor, Angiopoietin-2, Immunohistochemistry, Dynamic expression.

Hypervascularit'y is one of the most notable features of hepatocellular carcinoma (HCC) and angiogenesis contributes to its malignant biological characteristics such as invasion and high rates of recurrence and metastasis (Tang *et al.*, 2012). Direct experimental evidence has shown that an avascular HCC tumor rarely grows to a size larger than 2~3 mm², but once a tumor becomes vascularised the progression of tumor growth and metastasis is rapid (Melloul *et al.*, 2012).

Angiogenesis is a very complicated network regulated by many angiogenic factors of HCC. Two central endothelium specific growth factor families coordinate vascular development-namely, vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) with microvascular density (MVD) alteration in microvessel angiogenesis, development and metastasis of HCC (Shen *et al.*, 2012). The incidence of VEGF expression was 63.9% in HCCs, 78.3% in non-encapsulated HCCs, and 90.9% in HCC with extrahepatic metastasis and correlated with MVD (Tazi *et al.*, 2011). The abnormal expression levels of VEGF in sera of HCC patients were directly correlated with the metastasis and recurrence of tumors. The high expression of

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VEGF and abnormality of tissue MVD are useful predictors for vascular invasion and metastasis of liver tumors (Cao *et al.*, 2012).

Angiogenesis is known to be essential to the survival, growth, invasion, and metastasis of tumor cells. VEGF are an important angiogenic factor regulating tumor angiogenesis, it is the most potent directly acting angiogenic factor that is dimeric 46kD glycoprotein that specifically stimulates endothelial cell proliferation and enhances vascular permeability (Wu and Liu, 2012). Its expression by tumors is closely related to tumorigenesis, metastasis, and prognosis of HCC. Ang is a novel family of angiogenic factors which regulates angiogenesis under both physiological and pathological conditions (Mahasiripanth *et al.*, 2012). There is increasing evidence that Ang-2 displays VEGF dependent modulation of angiogenesis (Yao *et al.*, 2004). Previous reports have demonstrated that a high expression of VEGF in tissue and sera of HCC patients (Yao *et al.*, 2005). However, a little of hepatic VEGF alteration in hepatocarcinogenesis has still not been defined. In the present study, we investigated the dynamic alterations of hepatic VEGF and malignant transformation of hepatocytes and antiangiogenic intervention on HCC formation.

MATERIAL AND METHODS

Hepatoma model and drug intervention

Eighty-four male Sprague-Dawley rats, weighing 120~160 g, provided by the Animal Center of Medical Experiments, Nantong University, China, were randomly divided into three groups: control ($n=12$, 6 rats \times 2), 2-fluorenyl acetamide (2-FAA; Sigma, USA) ($n=36$, 6 rats \times 6), and 2-FAA plus thalidomide ($n=36$, 6 rats \times 6). The rats in the control and 2-FAA groups were treated according to a previously reported method. The rats in the 2-FAA plus thalidomide group were given food containing 2-FAA (0.05%), and simultaneously 100 mg/kg of thalidomide (No. 0710081, Changzhou Pharmaceutical Co., China) dissolved in olive oil administered intragastrically from the next day. Six rats in the 2-FAA group or in the 2-FAA plus thalidomide group and two rats in the control group were sacrificed every two weeks by anesthesia with diethyl ether. Blood was drawn from the heart. Parts of liver specimens were used for pathological and

immunohistochemical analysis or extraction of total RNA, and the rest were kept at -80 °C until use. All procedures were conducted in accordance with the Guidelines for Experimental Animals approved by the Animal Care and Use Committee of Nantong University, China.

The rat liver tissues fixed in 10% formalin solution were made the slices (4 μ m) after the dehydration, transparent and paraffin embedding. The slices were made the blank glass after the process of unfold, paste and roast. Histological examination was performed with H&E staining and pathological examination.

VEGF Immunohistochemistry

The streptavidin-peroxidase (S-P) method according to standard procedures and monoclonal antibody of rabbit anti-rat VEGF were purchased from Fuzhou Maixin Biotechnology Development Company, China. Liver specimens were fixed in 10% neutral formalin, embedded in paraffin and cut into 4 μ m of thick slices. After immunostained, restained with hematoxylin and dehydrated in a series of ethanol solution, and covered with neutral gum. The negative control included 0.01 Mol/L PBS instead of the primary and the secondary antibodies and S-P agent. Mastocancerous tissues with positive expression of VEGF were served as positive control. VEGF positive expression was brown particles in tissues. Expression of VEGF in hepatic tissues was evaluated on the base of the percentage of positive cells and classified as follows: weakly positive (+), when positive cells accounted for 10%~25% of the total cells; moderately positive (++), 26%~75%; and strongly positive (+++), >75%.

Detection of total protein in liver tissues

The liver tissue protein extraction kit (PMSF method) and the protein detection kit (BCA method) were bought from the Beyotime Institute of Biotechnology. 100mg rat liver tissues were taken and cut into fragments, mixed with extraction reagent according 1:20, added penylmethyl sulfonyl fluoride (PMSF), the tissues homogenate were compounded on ice, shake, centrifugation and so on, the supernates were transferred into a tube from the homogenates and were detected the protein concentrations by BCA-based assay.

Western blotting

The liver tissues were lysed in SDS sample buffer, separated with 10% SDS-acrylamide

gel, and electrotransferred to nitro- cellulose membranes. After blocking with 5% nonfat dry milk in TBST buffer [10 mMol/L Tris-HCl (pH8.0), 150 mMol/L NaCl, 0.05% Tween 20], the membranes were probed with anti- VEGF (1: 500; Maixin Biotechnology Development Company, Fuzhou, Chain), followed by incubation with HRP-conjugated antirabbit immunoglobulin G secondary antibodies (1: 2,000; Maixin Biotechnology Development Company, Fuzhou, Chain). The antibody binding was then visualized with enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology, Shanghai, China), and the band images detected with the ChemiDoc XRS+ system (Bio-Rad, USA) were densitometrically analyzed using the Quantity One (Ver. 4.62, Bio-Rad, USA).

Quantitative detection of VEGF and Ang levels

The level of VEGF or Ang was detected by using rat VEGF or Ang ELISA kit (ADL Biotech Dev Co., USA) according to the instruction of assay. 100 µl of goat anti-VEGF or anti-Ang polyclonal antibody (25 µg/ml in 0.1 Mol/L NaHCO₃) was put into each well of a 96-well ELISA plate, and incubated for 36 hours at 4°C. Nonspecific protein binding was blocked by 100 µl of bovine serum albumin (10 ml/L). Then 100 µl of sera or suspension of rat liver, added recombinant human VEGF or Ang for 1 hour at 37°C, then added 100 µl of rabbit anti-VEGF or anti-Ang polyclonal antibody (1: 200 dilution) for 1 hour at 37°C, added 100 µl of HRP-conjugated goat anti-rabbit immunoglobulin (1: 2000 dilution) for 1 hour at 37°C, developed with orthophenylene diamine, and

absorbance of each sample was detected at 490 nm. The concentration of VEGF or Ang was calculated according to a standard curve.

Statistical Analysis

Data was expressed as the mean ± standard deviation (SD). Statistical analyses were done using the SPSS10.0 software package. Differences between groups were assessed using Fisher’s exact test or the Chi-square test. P d” 0.05 was regarded as statistically significant.

RESULTS

Pathological alterations and occurrence of HCC

The comparison of rat liver morphological changes in the 2-FAA group is shown in Fig. 1. At the early stage, the structure of liver lobules was intact, with only a few hepatocytes showing punctiform denaturation and necrosis (50%, 18/36 vs. 86.1%, 31/36, Fig. 1, A1~2). At the middle stage, the structure of liver lobules was still basically intact, without large flake-like necrosis, and some hepatocytes proliferated mildly (25%, 9/36 vs. 13.9%, 5/36, Fig.1 B1~2). At the final stage (Fig.1, C1~2), the structure of liver lobules was still present in most hepatocytes, nodular hyperplasia was observed in some areas, and interestingly, no cancer was found in the 2-FAA plus thalidomide group (25%, 9/36 vs. 0%, 0/36, Table 1), where the structure of liver lobules was damaged in only a few rats, whose hepatocytes showed multi-nodular hyperplasia. And only one rat generated atypical hyperplasia. A significant difference was found between the 2-FAA group and the 2-FAA plus

Table 1. Expression and intensity of liver VEGF between the 2-FAA group and the 2-FAA+thalidomide group

Group	n	Positive (%)	VEGF intensity				Fisher	
			+	++	+++	++++		
Control	12	3(25)	9	3	0	0	0	
2-FAA								
Degeneration	18	16(89.0)	2	6	9	1	0	0.078
Precanceration	9	9(100)	0	3	4	2	0	0.044*
Hepatoma	9	9(100)	0	0	4	3	2	<0.001*
2-FAA+thalidomide								
Degeneration	31	23(74.2)	4	11	8	0	0	
Precanceration	5	5(100)	0	3	1	0	0	<0.001*
Hepatoma	0	0 (0)	0	0	0	0	0	<0.001*

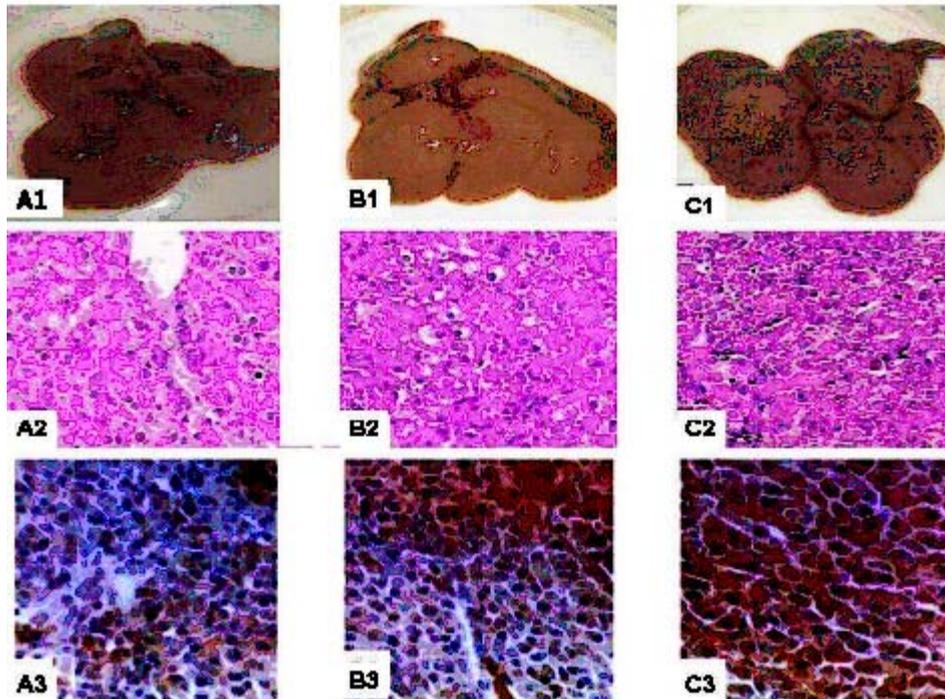
* Fisher value, compared with the control group.

thalidomine group ($P < 0.01$), suggesting that the antiangiogenic drug delayed the occurrence of HCC, as confirmed by pathological examination. Hepatic GGT expression as a control confirmed

the malignant transformation of rat hepatocytes (Fig.1 A3,B3,C3).

Expression of VEGF in hepatocarcinogenesis

Hepatic VEGF was expressed in the



A1,B1,C1, the appearance of liver tissues was observed in rat hepatocarcinogenesis. A1, at the early stage; B1, at the middle stage; and C1, at the late stages.

A2,B2,C2, the morphological alteration of hepatocytes was examined by H&E staining in rat hepatocarcinogenesis.

A2,B2,C2, the corresponding histopathological confirmation, hepatocyte degeneration, precanceration, and HCC (original magnification $\times 100$), respectively.

A3,B3,C3, the immunohistochemical staining of hepatic gamma-glutamyl transferase (GGT) expression was detected in rat hepatocarcinogenesis as control. A3,B3,C3, the corresponding hepatic GGT expression was observed with the slight positive staining at the stage of hepatocyte degeneration, the middle intensity at the stage of hepatocyte precanceration, and the overexpression at the stage of HCC formation (the streptavidin-peroxidase method, original magnification $\times 200$), respectively

Fig. 1. Appearance of rat liver and morphological changes of rat hepatocytes in hepatocarcinogenesis

cytoplasm of both sinusoidal cells and malignant hepatocytes in early experimental HCC tissues. Yellow-brown particles were seen on the rough endoplasmic reticulum and the mitochondrion with only a few in cells nuclear and none in cell membrane in malignant hepatocytes (Fig.2,A1~A4). The VEGF positive expression heightened gradually Along with the occurrence of rat hepatocytes malignant transformation. The incidences of rat liver VEGF expression was 50% in normal controls, 89% in degeneration rats, 100% in precancerous rats, and 100% in HCC rats, respectively. Moreover the VEGF

expression intensity assumed the enhancement tendency, and the liver tissues showed weakly positive results in the control group, strong immuno-staining of VEGF in the cytoplasm of sinusoidal cell in paracancerous liver tissues and the malignant hepatocytes in HCC tissues ($P < 0.05$, Table.1).

The liver VEGF expression in hepatocarcinogenesis was analyzed by Western blotting were up-regulated clearly during HCC development (Fig 2 B). VEGF protein levels increased significantly with advancing tumor

progress. The VEGF levels in rat tissues of pre-cancerous and HCC group were significantly greater than those of normal control and degeneration group ($P < 0.01$ Fig 2 C).

Alteration of VEGF levels in livers and sera

The expression levels of rat hepatic cytoplasm assumed the increasing tendency along with the liver histomorphological changes during development of HCC. In precancerous group and HCC group, the hepatic cytoplasm VEGF average

levels were higher than that in normal group, and the differences were significant ($P < 0.01$). The hepatic cytoplasm VEGF level in degeneration group was also higher than normal group but the difference was not statistically significant. After liver VEGF released into blood, a similar raised variation of VEGF levels in rat serum was found. Moreover, there was positive correlation of VEGF levels between in sera and in hepatic tissues ($r = 0.79$, $P < 0.01$). (Table.2)

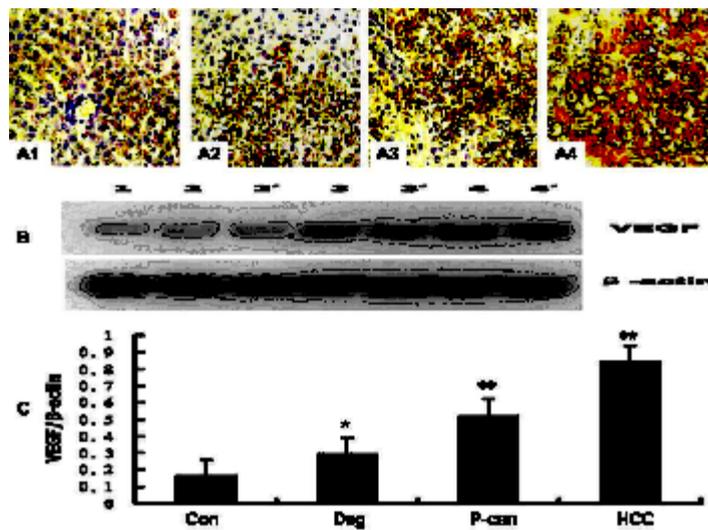


Fig. 2. Immunohistochemical staining with anti-VEGF and the confirmation by Western blot analysis in rat hepatocarcinogenesis
A, the VEGF-positive expression in cytoplasm and membrane of rat hepatocytes was examined by immunohistochemistry (the streptavidin-peroxidase method, original magnification $\times 100$). A1, the control rats; A2, the degeneration rats; A3, the precancerous rats; and A4, the HCC rats.
B, Western blotting of hepatic VEGF protein at the different stages of hepatoma formation. Lane 1, control rats; Lane 2, 2', degeneration rats; Lane 3, 3', precancerous rats; Lane 4, 4', HCC rats; and liver β -actin as control.
C, the relative ratio of liver VEGF expression to β -actin of rat livers was analyzed at the different stages of HCC formation. The data are means of independent triplicate experiments. Statistically significant compared with the control group: * $P < 0.05$; ** $P < 0.001$.

Fig. 2. Immunohistochemical staining with anti-VEGF and the confirmation by Western blot analysis in rat hepatocarcinogenesis

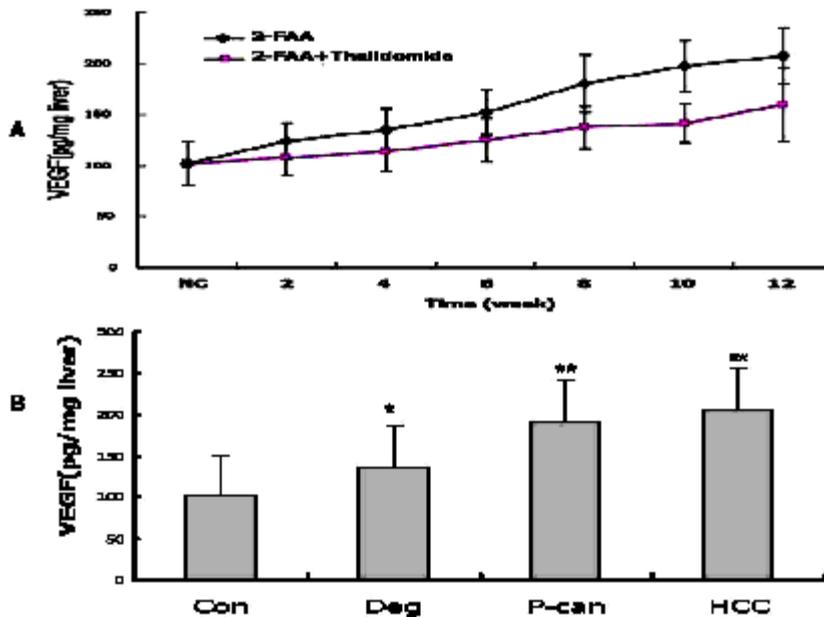
Expressions of Ang-2 in hepatocarcinogenesis

The dynamic alterations of serum VEGF and Ang levels during HCC progress are shown in Table 4. Both VEGF and Ang in precancerous group and HCC group were all significantly higher than those in normal and the relative values were in positive correlation ($r = 0.85$, $P < 0.01$, Table 3).

Inhibition of VEGF expression by antiangiogenic drug

The dynamic expression of hepatic VEGF

between the 2-FAA plus thalidomide group and the 2-FAA group are shown in Fig. 3. The expression of hepatic VEGF in the 2-FAA plus thalidomide group was inhibited during the development of hepatoma. The expression intensity of VEGF in the 2-FAA plus thalidomide group was significantly lower than that in the 2-FAA group ($\div 2 = 8.024$, $P < 0.001$), indicating that the antiangiogenic drug down-regulated VEGF expression and decreased angiogenesis.



A, the alteration of liver VEGF expression in hepatocarcinogenesis was quantitatively detected between the 2-FAA group and the 2-FAA+ thalidomide group.
 B, the expression of liver VEGF in hepatocarcinogenesis was quantitatively detected in the 2-FAA group according to the histopathological examination. Statistically significant compared with the control group: * $P < 0.05$; ** $P < 0.001$

Fig. 3. Dynamic expression and comparative analysis of VEGF specific concentration (pg/mg liver) in hepatocarcinogenesis

Table 2. Dynamic quantitative analysis of VEGF expression levels in livers and sera during rat HCC development

Group	n	Serum VEGF (pg/mL)		Liver VEGF(pg/mL)	
		Mean±SD	P value	Mean±SD	P value
Normal	6	11.91±2.32		101.14±20.71	
Deg	18	22.01±7.75	0.054	136.41±24.92*	0.032
Pre-can	9	30.20±7.73*	0.001	191.29±27.34*	0.000
HCC	9	38.22±9.12*	0.000	206.07±19.79*	0.000

P value* VS. Normal group, $P < 0.05$ or $P < 0.01$

Table 3. Relationship between VEGF and Ang in rat serum during HCC development

Group	n	Serum VEGF (pg/mL)		Liver VEGF(pg/mL)	
		Mean±SD	P value	Mean±SD	P value
Normal	6	11.91±2.32		105.23±19.23	
Deg	18	22.01±7.75	0.054	140.17±25.16*	0.031
Pre-can	9	30.20±7.73*	0.001	203.75±23.83*	0.000
HCC	9	38.22±9.12*	0.000	222.39±23.31*	0.000

P value* VS. Normal group, $P < 0.05$ or $P < 0.01$

DISCUSSION

Carcinogenesis of HCC is a multi-factor, multi-step, and complex process, and many genes such as proto-oncogenes, tumor suppressor genes, apoptosis genes, and growth factor genes have been implicated; apoptosis genes may play an important role in the process of HCC (Chen *et al.*, 2012). The exact mechanism of tumorigenesis in HCC remains unclear, but active neovascularization of the tumor is likely to play an important role (Balzarini *et al.*, 2012). Several studies have shown that HCC is a distinctively hyper-vascular tumor in clinical practice. Among the pro-angiogenic factors, VEGF is the most intriguing with regard to the Angiogenic process (Yan *et al.*, 2012; Kong *et al.*, 2012). In HCC it has been reported that VEGF was highly expressed in tumor lesions compared with the adjacent non-cancerous lesions, and overexpression of VEGF significantly enhanced HCC tumor development and angiogenesis (Inoue *et al.*, 2012). In our study, the histopathological changes in rat liver during the development of HCC, the expression levels of VEGF protein in rat hepatic tissues and sera were investigated by different immunological methods.

The morphological changes of rat livers were obviously observed during rat HCC development. Histological examination confirmed the rat hepatocytes from granule-like degeneration to atypical hyperplasia and HCC development after induced by 2-FAA (Fig1), synchronously, the progressing increasing of the expression levels of VEGF in hepatic tissues and sera during the course (Table1, Fig2). Immunohisto-chemical staining showed an increased expression level and ratio of VEGF in tumor hepatocytes and progressed with the dedifferentiation of HCC. VEGF expression was always present in the extracellular matrix, supporting the hypothesis of paracrine activation of VEGF at the level of tumor stroma. Consequently, increased VEGF expression might be responsible for the activation of angiogenesis in HCC. In this study, ELISA quantitative analyses and Western blotting all showed that VEGF expression levels increased progressively in rat liver tissues and sera during the development of HCC, and there was a positive correlation between the serum VEGF levels and the tumor VEGF expression, suggesting that the serum VEGF levels at least in part reflects the

tumor VEGF expression. It is worthwhile to note that the raise of serum VEGF levels already appeared in degeneration group. Thus, the overexpression of VEGF would well be a early event of hepatocarcinogenesis.

As we know, the angiopoietins, which also are specific for the vascular endothelium, have been identified (Lirdprapamongkol *et al.*, 2012). Previous studies have demonstrated that VEGF in association with Ang constitute a system that regulates vascular quiescence and endothelial plasticity, through which a balanced state of vascular maturity and development of complex vascular networks can be achieved (Takino *et al.*, 2012; Choi *et al.*, 2012). In our study, there were similar characteristics and trend of Ang in rat sera to that of VEGF during the development of HCC that suggested continuous Ang overexpression in the presence of VEGF would lead to maturation of tumor vessels, thus contributing to HCC development and Angiogenesis.

HCC is one of the most common malignancies worldwide, but treatment outcomes have remained generally poor (Martin- Padura *et al.*, 2012). In China, about 90% of HCC cases are associated with HBV infection. The up-regulation of VEGF is involved in the initiation, generation, and development of tumors, and is up-regulated in malignancies associated with inflammation (Wang *et al.*, 2012). In this study, we intra- gastrically administered an anti-angiogenic drug to intervene in VEGF expression, and assessed its influence on malignant transformation of hepatocytes in hepatocarcinogenesis. The liver tissues of rats fed with 2-FAA showed vacuole-like denaturation at the early stage, subsequent appearance of dysplastic nodules at the middle stage, and finally progressed to tubercles of cancerous nests. VEGF expression in the denatured, precancerous, and cancerous groups was significantly higher than in the control group. VEGF and finally contributed to hepatocarcinogenesis, indicating that VEGF activation was dynamically increased and might play an important role in HCC development.

Thalidomide is a glutamic acid analogue and has been investigated in the anti-angiogenic therapy of HCC (Andersen *et al.*, 2012; Tan *et al.*, 2012). In the 2-FAA group, the hepatocytes showed large flake necrosis at week 2. Then a remarkable compensatory hyperplasia occurred and the

structure of liver lobules was severely damaged. Precancerous and cancerous lesions started to appear at week 8. In the thalidomide group, before week 8, only a little focal necrosis occurred, and the structure of liver lobules was basically intact. At week 10, mild and local nodular hyperplasia appeared, and the structure of liver lobules was still basically intact. And at week 12, more nodular hyperplasia and a little atypical hyperplasia formed, and the structure of the liver lobules was partly destroyed. The density of VEGF expression in the 2-FAA plus thalidomide group was lower than that in the 2-FAA group, suggesting that the intervention of thalidomide in hepatocarcinogenesis effectively inhibits or delays the angiogenesis, hyperplasia, and cancer development of hepatocytes.

In conclusion, the expression of VEGF increased stepwise during the early stage of carcinogenesis. There would be a regulated network composed of VEGF and Ang that control angiogenesis of HCC. The over expression of Ang and VEGF caused neovascularization and induced HCC at last, and simultaneously, the expression levels of VEGF and Ang also reflected the pathological change degree of rat liver. Hepatic VEGF may participate in angiogenesis of hepatocyte canceration and Thalidomide, a antiangiogenic drug could inhibit the angiogenesis progress. Detection of VEGF expression during HCC development could be a useful marker for early diagnosis of HCC.

ACKNOWLEDGEMENTS

The study was supported by grants-in-aid from the Project of Jiangu Health (BL2012053), the project (K2010026) of Nantong Applied Research, the Int. Science & Technology Cooperation Program (S2012ZR0031) and the Postdoctoral Science Foundation Program (2012M521108) of China.

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