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• *BASIC RESEARCH* •

Fumagillin treatment of hepatocellular carcinoma in rats: An *in vivo* study of antiangiogenesis

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Abstract

AIM: To investigate the effect and possible mechanisms of antiangiogenesis therapy for HCC in rats.

METHODS: Adult male LEW/SsN rats were divided into 3 groups, 25 animals each. Group A was the control group. Groups B and C were given diethylnitrosamine, 5 mg/kg/d. In addition, group C rats received an intraperitoneal injection of fumagillin, 30 mg/(kg·d). Five animals in each group were killed at 6th, 12th, 18th, 20th and 24th wk to evaluate the development of HCC and metastasis. Weight of the rats, liver tumors, and number of organs involved by HCC were measured at each stage. We compared methionine aminopeptidase-2 (MetAP-2) mRNA, Bcl-2 mRNA, telomerase mRNA, and telomerase activity at 24th wk in the liver tissue of group A rats and tumor tissue of HCC from group B and C rats.

RESULTS: No HCC developed in group A, but tumors were present in group B and C rats by the 18th wk. At wk 20 and 24, the median liver weight in group B was 0.64 g (range: 0.58-0.70 g) and 0.79 g (range: 0.70-0.90 g) (P = 0.04), and that in group C was 0.37 g (range: 0.35-0.42 g) and 0.39 g (range: 0.35-0.47 g) (P = 0.67). The liver weight in group C rats was significantly lower than that in group B rats (P = 0.009). At the same time, the median metastasis score (number of organ systems involved) was 3 (range 2-3) in group B, and 1 (range 1-2) in group C, a significant difference between the groups (P = 0.007, 0.004). The levels of MetAP-2 mRNA were significantly higher in groups B and C than in group A (P = 0.025), and significantly higher in group C than in group B (P = 0.047). The level of Bcl-2 mRNA was significantly higher in group B than in group A (P = 0.024), but lower in group C than in group B, although not significantly (P = 0.072). Telomerase mRNA was significantly higher in group B than in group A (P = 0.025), but significantly lower in group C than in group B (P = 0.016). The same inter-group relationship was also true for telomerase activity (P = 0.025 and 0.046).

CONCLUSION: Fumagillin effectively inhibits both liver tumor growth and metastasis in rats *in vivo*. A possible mechanism is fumagillin-induced inhibition of MetAP-2, which plays an essential role in endothelial cell proliferation. Inhibition of MetAP-2 also results in inhibition of Bcl-2 and telomerase activity.

Key words: Hepatocellular carcinoma; Antiangiogenesis therapy; Fumagillin; MetAP-2

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INTRODUCTION

Hepatocellular carcinoma (HCC), a leading cause of death in Taiwan and many Asian countries, is difficult to treat because of early progression and metastasis. It is well known that angiogenesis is essential for the survival, growth, and metastasis of tumor cells[1-5]. Angiogenesis, formation of new blood vessels from the existing vascular bed, is a complex multistep process. There is extracellular matrix remodeling and binding of angiogenic factors to specific endothelial cell (EC) receptors, which results in EC proliferation, invasion of basement membrane, migration, differentiation, and formation of new capillary tubes. Their anastomoses develop a vascular network. There is much interest in inhibiting angiogenesis as a treatment strategy[6-9].

Fumagillin and its derivatives, such as TNP 470, are well-known antiangiogenic agents[9-18]. There are few published studies, however, of the *in vivo* effects of these agents on experimentally induced HCC in an animal model. The aim

of this study was to evaluate the therapeutic effect and possible mechanisms of antiangiogenesis in a rat model of HCC.

MATERIALS AND METHODS

Animals

Pathogen-free adult male LEW/SsN rats at the age of 8 wk were purchased from the National Science Council, Taiwan. They were fed standard diet chow pellets and water *ad libitum*. The study was begun when the rats were 12 wk old, and their median body weight (BW) was 372.5 g (range: 350-394 g). All experiments were performed according to standard guidelines for animal experiments and approved by the Animal Ethics Committees of Mackay Memorial Hospital.

Treatment of rats

The 75 rats were divided into 3 groups, 25 each. Group A rats were used as controls, receiving food only and no medication. To induce hepatocarcinogenesis, groups B and C rats were given diethylnitrosamine (DEN) ($C_4H_{10}N_2O$) (Sigma Chemical, St. Louis, MO. USA) in water at a dose of 5 mg/(kg·d). Group C rats received, in addition to DEN, intraperitoneal injections of fumagillin ($C_{26}H_{34}O_7$) (Sigma Chemical, St. Louis, MO, USA) 0.3 mg/(kg·d) beginning at the 18th wk of DEN induction.

Gross and histologic examination

Five rats in each group were sacrificed at 6th, 12th, 18th, 20th and 24th wk to evaluate the development of liver tumors and their changes. We measured the body weight, whole liver weight, and the number of involved organ systems of each rat. Liver and HCC specimens were examined by pathologists. The tumor weight was estimated by subtracting the liver weight of group A rats from that of group B or C rats. In examining for metastasis, we gave a score of 1 for HCC limited to the liver, 2 for extrahepatic extension or metastasis within the peritoneal cavity, and 3 if there were both intraperitoneal and lung metastases.

Molecular mechanisms

To investigate the molecular mechanisms of HCC inhibition by fumagillin, we detected methionine aminopeptidase 2 (MetAP-2) mRNA, Bcl-2 mRNA, and telomerase mRNA and telomerase activity from samples of the resected livers of group A and the resected tumors of groups B and C, in the 24th wk of treatment. GAPDH mRNA was used as a control.

Extraction of RNA

We homogenized resected tissue completely in 1mL of RNA-Bee™ (Tel-Test, Protech Technology Enterprises Co., Ltd, Friendswood, TX), added 0.2 mL chloroform, and shook vigorously for 15-30 s. We stored the samples on ice for 5 min and then centrifuged at 12 000 *g* for 15 min. We transferred the supernatant to a new 1.5 mL Eppendorf tube and precipitated the solution with 0.5 mL of isopropanol for 5 min at 4 ℃.We centrifuged the tube at 12 000 *g* for 5 min at 4 ℃ before removing the supernatant and washed the RNA pellet with 1 mL of isopropanol, shook to dislodge the pellet from the side of the tube. We centrifuged the pellet again at 12 000 *g* for 5 min at 4 ℃, removed the supernatant, and washed the RNA pellet once with 75% ethanol, shook to dislodge the pellet from the side of the tube. We suspended the pellet in at least 1 mL of 75% ethanol and centrifuged it at 7 500 *g* for 5 min at 4 ℃ before carefully removing the ethanol. The RNA was to air dried and then dissolved in DEPC-H_2O (50-100 μL) and stored at -80 ℃.

Reverse transcription

We heated the RNA sample at 55 ℃ for 10 min, chilled it on ice, and then added the following reagents: 4 μL 5 XRT buffer containing Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, and 10 mmol/L DTT (dithiothreitol); 3 μL 10 mmol/L dNTP (deoxyribonucleoside triphosphate); 1.6 μL Oligo-d $(T)_{18}$ and 0.4 μL random hexamers (N)6 (1 ug/μL); 0.5 μL RNase inhibitor (40 units/μL); 3 μL 25 mmol/L $MnCl_2$; 6 μL RNA in DEPC-H_2O; and 0.5 μL DEPC-H_2O. We incubated the mixture at 70 ℃ for 2 min and then chilled it to 23 ℃ to anneal the primer to the RNA. We added 1 μL of M-MLV RTase (Moloney murine leukemia virus reverse transcriptase, 200 units/μL, Promega) and incubated it for 10 min at 23 ℃ followed by 60 min at 40 ℃. We then heated it at 94 ℃ for 5 min, chilled it on ice, and stored the cDNA at -20 ℃.

By PCR amplification of MetAP-2, Bcl-2, telomerase, and GAPDH cDNA

First-strand cDNA synthesis was carried out using 2 μg of total RNA purified from 50 mg tissue. Reverse transcription was performed in a 20 μL final volume containing 2 μg of random hexamer (Gene Teks Bioscience Inc., Taipei), and 1.5 mmol/L each of dATP, dCTP, dGTP, and dTTP. Each reaction mix was incubated for 8 min at 23 ℃ with 20 U of rRNasin (RNase inhibitor; Promega, Madison, WI) followed by incubation with 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Paisley, UK) for 60 min at 40 ℃ followed by 5 min at 94 ℃. PCR was performed in a final volume of 50 μL, by using 2 μL of cDNA solution in a mix containing 0.4 mmol/L deoxynucleotide triphosphates, 40 pmol of both sense and antisense oligonucleotide primers according to the MetAP-2, Bcl-2 and telomerase type to be detected, 2.5 mmol/L $MgCl_2$, 2.5 U of Taq DNA polymerase (Promega) and 5 μL of 10 min Taq DNA polymerase reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl [pH9.0], 1% Triton-X-100). PCR primer sequences of the sense and antisense oligonucleotides for MetAP-2, Bcl-2 and telomerase, as well as the direction, size and reaction conditions are shown in Table 1. For example, the MetAP2 phosphorothioate anti-sense oligonucleotide (5'-AGTATTT ACTTTCTCCCAAG-3') and its relative scrambled sequence (S'-CTTGG GAGAAAGTAAATACT- 3') were synthesized by Sigma-Genosys Ltd, Woodlands, TX, USA. The anti-sense start position on the MetAP2 mRNA coding region was 1 284. This region corresponds to the large helical domain insertion on the surface of the type 2 isozyme. GAPDH was used as a control, with the quantities of the other mRNA products reported as a fraction of their intensity compared

Table1 Sequence of sense and antisense primers in reverse transcription-polymerase chain reaction (RT-PCR) analysis for MetAP-2, Bcl-2 and telomerase mRNA expression

Name	Sequence	Direction	Expected product size (bp)	PCR conditions for pair of primers
MetAP-2-S	TGG CGG GCG TGG AAG AGG	Sense	282	1 cycles: 94 ℃, 7 min 50 cycles: 94 ℃, 40 s; 54 ℃, 40"; 72 ℃, 1 min
MetAP-2-AS	GCA CCA TCA CCA TCA CCA TCT CC	Antisense	282	1 cycle: 72 ℃, 10 min; 4 ℃ overnight
Bcl-2-S	AGA TGA AGA CTC CGC GCC CCT CAG G	Sense	566	1 cycles: 94 ℃, 7 min 50 cycles: 94 ℃, 40 s; 54 ℃, 40"; 72 ℃, 1 min
Bcl-2-AS	CCA GGT ATG CAC CCA GAG TGA TG	Antisense	566	1 cycles: 72 ℃, 1 min; 4 ℃ overnight
Telomerase-S	GAC ATG GAG AAC AAG CTG TTT GC	Sense	185	1 cycles: 94 ℃, 7 min 50 cycles: 94 ℃, 40 s; 54 ℃, 40"; 72 ℃, 1 min
Telomerase-AS	ACA GGG AAG TTC ACC ACT GTC	Antisense	185	1 cycle: 72 ℃, 10 min; 4 ℃ overnight
GAPDH-S	ACC ACA GTC CAT GCC ATC AC	Sense	485	1 cycles: 94 ℃, 7 min 50 cycles: 94 ℃, 40 s; 54 ℃, 40"; 72 ℃, 1 min
GAPDH-AS	TCC ACC ACC CTG TTG CTG TA	Antisense	485	1 cycle: 72 ℃, 10 min; 4 ℃ overnight

to GAPDH mRNA. To eliminate any possibility of genomic DNA contamination, PCR amplification reaction was carried out on each sample for RNA extraction. As another internal contamination control, PCR amplification was also carried out on a sample of reaction mixture in the absence of cDNA.

Telomeric repeat amplification protocol (TRAP) assay

Either an unamplified conventional standard or a polymerase chain reaction-ELISA-based assay (Roche Molecular Biochemicals, Foster City, CA) was used to measure telomerase activity. Cell equivalents (1×10^3 to 5×10^3) were used to visualize the DNA ladder according to the standard protocol. For polymerase chain reaction-ELISA, 2×10^3 cell equivalents were used. The polymerase chain reaction-ELISA protocol was provided by the assay kit manufacturer (Roche Molecular Biochemicals). Each set of TRAP assays included control reaction tubes without any extract or with RNase A (200 μg/mL)-treated extracts. To quantify the levels of telomerase activity, the average densitometric optical density of the first six TRAP bands after a primer band was reported as a ratio of the internal TRAP assay standard band.

Quantification of telomerase activity

After the TRAP reaction, hybridization and ELISA, the level of telomerase activity in a given sample was determined by comparing the signal from the sample to the signal obtained using a control template (TS8; solutions 4 or 5). The control templates provided with the TeloTAGGG telomerase PCR ELISA^{plus} are ready-to-use solutions containing TS8 at a concentration of 0.001 mol/mL and 0.1 mol/mL. The control templates used were identical to a telomerase elongation product with 8 telomeric repeats. However, because amplification of the TRAP products and the internal standard (IS) are competitive, the signal of the internal control might be near background level when analyzing samples with very high telomerase activity.

Relative telomerase activities (RTA) within different samples were obtained using the following formula:

$$RTA = \frac{(A_S - A_{SO})/A_{S,IS}}{(A_{TS8} - A_{TS8,0})/A_{TS8,IS}} \times 100$$

A_S: absorbance of sample; $A_{S,0}$: absorbance of heat- or RNase-treated sample; $A_{S,IS}$: absorbance of internal standard (IS) of the sample; A_{TS8}: absorbance of control template (TS8), $A_{TS8,0}$: absorbance of lysis buffer; $A_{TS8,IS}$: absorbance of the internal standard of the control template.

Statistical analysis

A statistical software package (SPSS for Windows, version 8.0, Chicago, IL) was used, with Student's *t* test for continuous variables and χ^2 or Fisher's exact test for categorical variables. Non-parametric data were analyzed with Mann-Whitney test or Kruskal-Wallis test. Significance was accepted at $P<0.05$.

RESULTS

Hepatocarcinogenesis

No tumor was found in the liver of group A rats at any time point in the study. All group B and C rats developed diffuse neoplasms in all lobes of the liver by the end of 18th wk. However, after fumagillin treatment, hepatic tumors in group C rats at wk 20 and 24 had necrosis and hemorrhage, no change was seen in group B. Both group B and C rats had a slight but insignificant increase in weight from wk 20 to 24; the difference in the changes between the two groups was also not significant ($P>0.05$, Table 2).

Table 2 Body weight of rats at wk 20 and 24

	Rats (n = 10)	Body weight (g)	
		20th wk	24th wk
Group B	DEN only	398	400
		[386-409]	[384-411]
Group C	DEN + Fumagillin	397	398
		[380-410]	[382-414]
P value		NS	NS

Weight: median [range]; *P* value: non-parametric test (Mann-Whitney *U* test)
Group A (control): 372 [350-380] g at wk 20 and 380 [351-394] g at wk 24.

The median tumor weights in group B at wk 20 and 24 were 0.64 g (range 0.58-0.70 g) and 0.79 g (0.70-0.90 g) respectively, a significant increase. Those in group C were 0.37 g (range 0.35-0.42 g) and 0.39 g (0.35-0.47 g). The tumor weight in group C rats was significantly lower than that in group B at wk 20 and 24 (P = 0.009, P =0.009, Table 3), suggesting that fumagillin inhibited the tumors.

Metastasis

Group B rats had a median metastasis score of 3 (range 2-3) at wk 20 and 24. Group C rats had a median score of 1 (range 1-2) at wk 20 and 24. The difference between the

two groups was statistically significant ($P = 0.007$, $P = 0.004$, Table 3).

Table 3 Inhibitory effect of fumagillin on hepatic tumor growth and metastasis in LEW rats at wk 20 and 24

Treatment groups		Liver tumor (B/C-A) (g, median)		No. of HCC-involved organs[1]	
		20th wk	24th wk	20th wk	24th wk
Group B	DEN only	0.64[1]	0.79[1]	3	3
	($n = 5$)	[0.58-0.70]	[0.70-0.90]	[2-3]	[3-3]
Group C	DEN + Fumagillin	0.37	0.39	1	1
	($n = 5$)	[0.35-0.42]	[0.35-0.47]	[1-2]	[1-2]
P value		0.009	0.009	0.007	0.004

Weight: median [range] of 5 rats in each step; *P* value: non-parametric test (Mann-Whitney U test); [1]organs involved: 1 (liver only), or 2 to 3 (lung or/and peritoneum, in addition to liver). Liver tumor (B/C-A): B rat liver tumor weight = liver weight of group B rat minus that of group A rat; C rat liver tumor weight = liver weight of group C rat minus that of group A rat.

Microscopic findings

All rats had evidence of HCC after 18 wk of treatment with DEN. However, after administration of fumagillin for 2 wk, tumors in group C rats had dilated bile ducts and sinusoids, and karyorrhectic changes of endothelial cells lining the sinusoids. After 6 wk of fumagillin treatment, multiple areas with varying degrees of necrosis and hemorrhage were found in tumors of the group C rats. Some cancer cells had membrane blebbing, cytoplasmic vacuolization, and mitochondrial body formation, and there were neutrophil and histolytic infiltration. These changes were not present in HCC of group B rats at wk 20 and 24.

Assays for MetAP-2, GAPDH, Bcl-2, and telomerase mRNA

The results of quantitative RT-PCR analysis are shown in Table 4. The median intensity of MetAP-2 mRNA (compared with GAPDH) in liver tissue of group A and HCC tissue of groups B and C at the 24th wk was 0.34 (range 0.32-0.36), 0.50 (0.33-0.70) and 0.58 (range 0.40-0.73) respectively. The groups all differed significantly from one another. Comparable results for Bcl-2 mRNA were 0 in group A (range 0-0.19), 0.45 in group B (range 0.22-0.63) and 0.38 in group C (range 0-0.32). Differences among the 3 groups and between groups A and C as well as between groups B and C were not significant. However, the difference between groups A and B was significant ($P = 0.024$). The median value of telomerase mRNA was 0.30 in group A (range 0.29-0.32), 0.43 in group B (range 0.35-0.47), and 0.34 in group C (range 0.29-0.37). The value for group B was significantly higher than that for group A or C ($P = 0.025$, 0.016), while the value did not differ significantly in groups A and C ($P = 0.655$).

Telomerase activity

The median telomerase activity of HCC tissue at wk 24 in the 3 groups was 47.6% (range 46-71%), 225.0% (187-310%), and 203.5% (94-292%) respectively. Telomerase activity in group A was significantly lower than that in group B ($P = 0.025$) or C ($P = 0.025$). The activity was significantly higher in group B than that in group C ($P = 0.046$, Table 4).

DISCUSSION

Our study has confirmed the inhibitory effects of fumagillin on HCC and allowed us to develop a model describing its effects both at tissue and cellular level and at molecular level. The advantage of our investigation is that it was an *in vivo* rather *in vitro* study or one, which used subcutaneously implanted tumors. Evaluating HCC progression and inhibition *in situ* in the liver can increase our understanding of the disease.

Tumor weight in the group B animals treated with DEN increased significantly from wk 20 to wk 24, confirming its effect on progression of HCC. However, in group C rats being given both DEN and fumagillin, the tumor weight was significantly lower than that in group B rats at wk 20 and 24. In addition, the fumagillin-treated rats had a lower metastasis score than those treated with DEN alone.

Fumagillin has been reported to cause weight loss[18,19], although Kin *et al*[20] found that liver weight as a function of body weight is actually higher in rats treated with fumagillin derivative TNP-470[20]. In our fumagillin-treated rats, insignificant weight loss was only at wk 20 and 24. We think that this is most likely due to the low dose we used. In addition, the tumor weight was determined by comparing whole liver weight with that of controls. Thus, fumagillin-induced body weight loss cannot explain the lower tumor weight in the fumagillin-treated rats.

It is proposed that the mechanism by which fumagillin

Table 4 Comparison of MetAP-2 mRNA, Bcl-2 mRNA, telomerase mRNA and telomerase activity among the 3 groups of rats at wk 24

Parameters	Group			*P* value		
	A ($n = 3$)	B ($n = 5$)	C ($n = 5$)	A *vs* B	A *vs* C	B *vs* C
MetAP-2 mRNA	0.34	0.50	0.58	0.025	0.025	0.047
(0.32-0.36)	(0.33-0.70)	(0.40-0.73)				
Bcl-2 mRNA	0	0.45	0.38	0.024	0.608	0.072
(0-0.19)	(0.22-0.63)	(0-0.42)				
Telomerase mRNA	0.30	0.43	0.34	0.025	0.655	0.016
	(0.29-0.32)	(0.35-0.47)	(0.29-0.37)			
Telomerase	47.6	225.0	203.5	0.025	0.025	0.046
Activity (%)	(46-71)	(187-310)	(94-292)			

Non-parametric test: Mann-Whitney and Kruskal-Wallis tests.

inhibits HCC is by inhibiting angiogenesis, specifically by blocking EC proliferation. By inducing apoptosis of ECs, vascularization is disrupted, leading to infarction of HCC.

It is well documented that fumagillin agents directly inhibit proliferation and migration of ECs *in vitro* and *in vivo* in various tumor models, such as in tumors implanted subcutaneously in mice[21]. Folkman[22] has stated that the goal of antiangiogenic therapy is to maximize apoptosis of ECs in tumor vascular beds. Fox *et al*[5] pointed out that this is a particularly attractive approach, as ECs are directly accessible through the blood and because they are 'normal' cells and therefore unlikely to become resistant to treatment. Similarly, according to Prox *et al*[23], fumagillin derivatives do not directly inhibit proliferation of pancreatic cancer cells, but they inhibit EC proliferation, increasing apoptosis of tumor cells by reducing microvessel density.

At 24 wk, our fumagillin-treated rats had massive hemorrhage and necrosis in the liver tumors, a finding not seen in the rats treated with DEN alone. Damage to the vessels supplying the tumor could certainly account for these changes, as ischemia can result in both apoptosis and necrosis of cancer cells. There is a critical difference between these two causes of cell death. Necrosis occurs when the cell suffers a major insult. Damage is generally so severe that the cell loses its ability to maintain membrane integrity, rapid swelling results in bursting of the membrane and release of its contents. This sets up a chain reaction, as toxic enzymes released from the dead cells attack surrounding cells. A wave of necrosis radiates out from the initial site of damage.

There is as yet no evidence that fumagillin can kill HCC cells. However, Catalano *et al*[24] noted that fumagillin might induce apoptosis by early mitochondrial damage in malignant mesothelioma cells. Yoshida *et al*[25] reported that fumagillin-like agents inhibit both the growth and migration of human hepatoma and vascular ECs *in vitro* and may suppress *in vivo* growth of hepatoma, associated with a reduction in the microvasculature and macrophage counts. The speculation that fumagillin inhibits not only ECs but also cancer cells to some degree warrants more studies.

Methionine aminopeptidases (MetAPs) are enzymes involved in the removal of N-terminal methionine from peptides and proteins. The molecular target of fumagillin is MetAP-2, which appears to be important in EC growth[26]. This enzyme's effects appear to include protein co-translational or posttranslational processing and myristoylation, as well as regulation of protein stability[27]. Sin *et al*[28] demonstrated that fumagillin selectively inhibits MetAP-2 protein *in vivo* by covalently binding to it and blocking its aminopeptidase activity. This would disrupt post-translational modification with failure of myristoylation, contributing to EC cytostasis, with arrest in the late G1 phase.

If MetAP-2 plays a comparable role in tumor cells, that would further support the hypothesis that fumagillin directly inhibits tumor cells. Studies have shown that *in vitro* exposure of human microvascular endothelial cells (HMVECs) to 1 nmol/L fumagillin for 24 h results in a two- to six-fold increase in MetAP2 protein in all cell types[29]. Up-regulation of *MetAP-2* gene thus seems to be a common phenomenon in cells treated with fumagillin. It is hypothesized that the loss of MetAP-2 catalytic function in cells exposed to fumagillin leads to up-regulation of the gene. This response might itself contribute to cytostatic inhibition of ECs, possibly via an excessive increase in the ribosomal regulatory ($p67$) function of increased MetAP-2 protein in its free form or bound to fumagillin.

DEN treatment significantly increased rat liver MetAP-2 mRNA over that in untreated rats in our study. We attribute this to rapid cell growth, and hence increased expression of MetAP-2 mRNA, during carcinogenesis. Fumagillin-treated rats had an even higher MetAP-2 mRNA level than those treated with DEN alone, a difference that achieved statistical significance ($P = 0.047$). With fumagillin inhibition, MetAP-2 mRNA in HCC probably first decreased and then increased to compensate for loss of MetAP-2 catalytic activity. However, it did not quite achieve a two-fold increase over the level in the control rats. This might be due to the fact that we used a relatively low dose of fumagillin.

MetAP2 also affects two key regulators of proliferation and programmed cell death, namely Bcl-2 and telomerase. Inhibition of MetAP-2 in mesothelioma cells reduces both mRNA and protein expression of the anti-apoptosis gene *bcl-2* as well as telomerase activity. This suggests a major role for MetAP2 in proliferative and apoptosis pathways[24,30,31]. However, the mechanism by which MetAP2 regulates *bcl-2* expression remains unknown. MetAP2 is not a transcription factor; therefore, it is unlikely that it directly regulates *bcl-2* gene expression. Instead, by its posttranslational processing effects, MetAP2 may alter the function of *bcl-2* transcription factors.

The *bcl-2* gene encodes a 26-kDa protein that protects cells against apoptosis in a variety of experimental systems. Bcl-2 maintains mitochondrial integrity by regulating the opening of the transition pore, thus preventing release into the cytosol of caspase activators. Furthermore, Bcl-2 protein prevents apoptosis by inhibiting lipid peroxidation of the cell membrane. It may therefore be important in protecting ECs against apoptosis as they are engaged in forming new vessels in tumors. It may also potentiate their differentiation into functional blood vessels. Second, Bcl-2 might potentiate the ability of ECs to differentiate. Some authors have reported that MetAP2 inhibition causes a time-dependent down-regulation of the *bcl-2* gene, whereas it does not alter expression of the pro-apoptotic gene, bax. Another mechanism for downregulation of *bcl-2* expression, at least in selected systems, is by an increase in p53 expression induced by fumagillin. In our study, Bcl-2 mRNA was significantly higher in DEN-treated rats than in controls. It was somewhat lower in the fumagillin-treated rats than in group B, but the difference was not statistically significant. It is possible this was because of our small sample number. Or, it is possible that down-regulation of *bcl-2* does not play a significant role in this particular model of tumor inhibition.

In addition to deregulation of apoptosis, it is increasingly clear that oncogenesis is driven by the activation of telomerase, a ribonucleoprotein complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes. Telomerase is thought to be responsible for cell immortality, primarily by protecting chromosomes from rearrangement. Telomerase activity is not detected in normal liver, but it has been detected in the vast majority of human

cancer cells. This has raised the possibility that telomerase may be an important target for therapy aimed at controlling cell growth. Kishimoto *et al*[31] emphasized the critical step of telomerase activation in hepatocarcinogenesis and tumor progression. Takahashi *et al*[32] reported that telomerase reactivation during hepatocarcinogenesis might be regulated only by hTERT, whereas increased telomerase activity in tumor progression might be regulated by both hTERT (reverse transcript) and hTERT (RNA component). Shimada *et al*[33] maintained that the higher the telomerase activity in HCC, the higher the malignant potential.

The relationship between Bcl-2 and telomerase activity remains controversial. Recent studies have shown an association between telomerase activation and Bcl-2 deregulation in a wide range of human carcinoma cells. Elkak *et al*[34] found that telomerase activity is higher in the Bcl-2-expressing cases of colorectal cancer than in Bcl-2-non-expressing cases, suggesting that Bcl-2 expression may be related to telomerase activity in colorectal carcinoma. Iida *et al*[30] and Elkak *et al*[34] hypothesized that telomerase reactivation in human breast cancer is associated with increased immunohistochemical expression of Bcl-2. Mandal *et al*[35] reported that the stable overexpression of Bcl-2 in human cancer cells with low Bcl-2 expression is accompanied with increased levels of telomerase activity. In low-grade tumors, Bcl-2 is inversely correlated with telomerase activity. Ohmura *et al*[36] suggested that the biological role of the Bcl-2 protein is altered by the degree of tumor aggressiveness, so that it works with telomerase against genetic instability. HCC is an aggressive malignancy, and we propose Bcl-2 and telomerase work together in this tumor. It is possible that MetAP2 acts as upstream of Bcl-2, while the Bcl-2 site of action is likely to be upstream of that of telomerase and caspases.

We quantified both telomerase mRNA and telomerase activity to ensure the accuracy of our results. DEN-treated rats had significantly higher values for both at 24th wk compared to control and fumagillin-treated rats. Telomerase mRNA in fumagillin-treated rats did not differ significantly from that in controls, although the telomerase activity remained significantly higher in group C than in group A. There are three possible explanations for this. First, as suggested by Kenmochi *et al*[37] and Ohta *et al*[38], differentiation may worsen as the tumor progresses; there may be localized spread of the tumor, including intrahepatic metastasis or portal vein thrombosis. Second, as the treated tumor necroses, regeneration of hepatocytes may increase telomerase activity. Third, it has been reported that telomerase may be expressed in lymphocytes. Lymphocytic infiltration may occur during tumor necrosis, so that the total telomerase activity we measured included a proportion generated by lymphocytes, thus overestimating that contributed by the tumor.

Our study demonstrated the ability of fumagillin to inhibit both progression of HCC in the liver itself and systemic metastasis *in vivo* in DEN-treated rats. We also examined three molecular targets of fumagillin in HCC. We found that HCC tissue in fumagillin-treated rats had a compensatory elevation of MetAP-2 mRNA after an initial decrease, with associated decreases in Bcl-2 mRNA, telomerase mRNA, and telomerase activity. These results may be attributed mainly to inhibition of ECs by fumagillin. A possible mechanism is that fumagillin-induced inhibition of MetAP-2 plays an essential role in EC proliferation. Inhibition of MetAP-2 also results in inhibition of Bcl-2 and telomerase activity.

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