

# Implications of Human Macrophage Metalloelastase and Vascular Endothelial Growth Factor Gene Expression in Angiogenesis of Hepatocellular Carcinoma

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## Objective

To determine molecular mechanisms involved in angiogenesis of hepatocellular carcinoma (HCC).

## Summary Background Data

Tumor angiogenesis is believed to derive from the balance between angiogenic stimulators and inhibitors. It has been suggested that the switch to the angiogenic phenotype requires both upregulation of the first and downregulation of the second. However, its molecular basis in vivo remains obscure. In this study the authors analyze the participation of two factors in angiogenesis of HCC—human macrophage metalloelastase (HME), a matrix metalloproteinase responsible for the generation of angiostatin, a potent angiogenesis inhibitor, and vascular endothelial growth factor (VEGF), the most potent endogenous angiogenic factor.

## Methods

Tumorous and contiguous nontumorous tissues from 25 patients with HCC who underwent curative partial hepatectomy were subjected to Northern blot analysis to detect HME and

VEGF messenger RNA (mRNA) expression. Western blot analysis was used to detect angiostatin. Tumor vascularity was evaluated using hepatic angiography.

## Results

Eleven of the 15 cases expressing the HME gene showed hypovascular tumors, whereas hypervascular tumors were seen in 9 of the 10 HME-negative cases. The median of HME mRNA expression (tumorous/nontumorous ratio) was 6.5 (range 0–264.5) in the hypovascular group and 0 (range 0–3.2) in the hypervascular group. A stepwise logistic analysis revealed that HME and VEGF mRNA expression were two independent variables significantly affecting the vascularity of HCC tumors.

## Conclusion

HME gene expression is significantly associated with hypovascular tumors; moreover, angiogenesis in HCC is not determined by a single factor, but depends on the net balance between HME and VEGF gene expressions.

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The growth of solid tumors requires new blood vessel formation to facilitate the delivery of nutrients and oxygen and the removal of catabolites, as has been experimentally demonstrated.<sup>1,2</sup> Thus, long-term suppression of

angiogenesis may become a therapeutic option for malignancies.<sup>3,4</sup> However, the mechanisms that control it are not completely understood. Recently, it has been proposed that a balance between endogenous angiogenic stimulators, including members of the vascular endothelial growth factor (VEGF), fibroblast growth factor, and platelet derived-endothelial cell growth factor families, among many others, and endogenous angiogenic inhibitors, such as angiostatin, endostatin, and thrombospondin, is a critical condition for the promotion of the neovascularization of tumors. Indeed, several reports

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have stated that the switch to the angiogenic phenotype requires both upregulation of the first and downregulation of the second.<sup>5,6</sup> These molecular bases, in association with the lack of detailed studies on this aspect in human tumors, encouraged us to explore possible molecules regulating tumor angiogenesis in hepatocellular carcinoma (HCC), which is generally considered to be a hypervascular tumor with an unsatisfactory long-term survival rate after surgical resection and conventional adjuvant chemotherapy.

The matrix metalloproteinase (MMP) family includes an increasing list of enzymes characterized by their ability to degrade extracellular matrix as well as nonmatrix substrates. Although MMPs have long been associated with tumor invasion and metastasis,<sup>7</sup> their association with angiogenesis remains controversial. In our recent report,<sup>8</sup> we demonstrated, for the first time to our knowledge, that human macrophage metalloelastase (HME), identified as MMP-12, and formerly isolated from alveolar macrophages of cigarette smokers,<sup>9,10</sup> can be directly expressed by some human HCCs (62.5%), and that its expression is strongly associated with the generation *in situ* of human angiostatin,<sup>11</sup> a potent inhibitor of angiogenesis. Previously, only one basic experimental approach had been reported, in which a mouse counterpart of HME produced by tumor-infiltrating macrophages was responsible for the generation of angiostatin from plasminogen, inhibiting the growth and metastasis of Lewis lung carcinoma in mice.<sup>12</sup>

Among angiogenic factors, VEGF is the most potent endogenous mediator of angiogenesis known.<sup>13,14</sup> VEGF is a diffusible endothelial cell-specific mitogen and angiogenic factor that also increases vascular permeability. These actions are mediated through two endothelial cell tyrosine kinase receptors, the *fms*-like tyrosine kinase and the kinase domain receptor.<sup>15,16</sup> Both VEGF and its receptors have been found to be expressed in a variety of tumors,<sup>17-19</sup> where overexpression of VEGF results in dramatically increased angiogenesis and subsequent tumor expansion, whereas the blockade of the VEGF receptors inhibits angiogenesis, leading to a reduction of new vessels, a higher degree of necrosis, and a reduction in size of certain tumors. In HCC, it has been demonstrated that 60% of these tumors exhibit high expression of VEGF, which significantly correlates with the hypervascularity of the tumors.<sup>20</sup>

We designed the present study to clarify the *in vivo* molecular role of HME gene expression in angiogenesis of HCC, and to examine whether VEGF expression can modify the angiogenic response of HME gene expression. The results presented here indicate that HME messenger RNA (mRNA) expression is significantly associated with hypovascular tumors and, moreover, that the final angiogenic phenotype of HCC tumors does not depend on a single factor, but on the net balance between HME and VEGF.

## PATIENTS AND METHODS

### Study Population

Twenty-five patients who had undergone curative partial hepatectomy for HCC at the Department of Surgery & Surgical Basic Science, Kyoto University Hospital, Kyoto, Japan, were included in this study. There were 21 men and 4 women with an average age of  $61.56 \pm 9.18$  (mean  $\pm$  SD; range 39–76). The central portion of the tumor without necrosis and contiguous nontumorous tissues were collected once we had informed consent. Specimens were macroscopically and histologically examined (Table 1) in accordance with the Classification of Primary Liver Cancer of the Liver Cancer Study Group of Japan<sup>21</sup> and stored at  $-70^{\circ}\text{C}$  until used. All samples were subjected to Northern blot analysis, to detect HME and VEGF mRNA expression, and Western blot, to detect angiostatin.

### cDNA Probes and Northern Blot Hybridization

Total RNA was isolated from tumorous and nontumorous liver tissues by the acid guanidinium-thiocyanate/phenol/chloroform method; and poly (A)<sup>+</sup> RNA was selected by Oligotex-dT30 (Takara, Japan). Northern blot hybridization was performed as previously described.<sup>8</sup> The probes used were 598 bp and 418 bp reverse transcription-polymerase chain reaction products corresponding to HME and human VEGF<sub>165</sub> respectively, and S26 ribosomal protein cDNA as internal control. The probes were prepared as described elsewhere.<sup>8,20</sup> HME and human VEGF<sub>165</sub> mRNA levels in the paired tumorous and nontumorous tissues were quantified by measuring the intensities of the appropriate bands in the autoradiograms (1.8 Kb band and 4.5 Kb band respectively) using an ATTO Densitograph (Tokyo, Japan) and were normalized to S26 ribosomal protein mRNA. Then, the tumorous/nontumorous (T/N) ratio of the former to the latter was determined (see Table 1).

### Angiostatin Detection

In all cases, Western blot analysis was performed to detect angiostatin by using murine monoclonal antibodies against kringle 1-3 elastase fragment of human plasminogen, subclass IgG1 (Product #P1517, Sigma Chemical Co., St. Louis, MO), as previously described.<sup>8</sup> The angiostatin levels in the paired tumorous and nontumorous tissues from the same patient were quantified by an ARGUS-50, 3.3 (Hamamatsu, Japan), and the ratio of the former to the latter was defined as the angiostatin T/N ratio (see Table 1).

### Angiographic Evaluation

The correlation of HME and HME/VEGF mRNA expression with the angiographic findings was studied in all 25 patients who underwent selective hepatic angiography. The

**Table 1. CLINICOPATHOLOGIC AND MOLECULAR FEATURES OF PATIENTS WITH HEPATOCELLULAR CARCINOMA**

Patient	Age (yr)	Sex	Tumor Size (cm)	Portal Involvement	Cirrhosis	Histologic Grade*	mRNA Expression (T/N Ratio)			Angiostatin†	Tumor Vascularity
							HME	VEGF	HME/VEGF		
1	47	M	4.6	+	With	Poor	264.5	1.06	+/+	1.11	Hypovascular
2	39	M	12	+	Without	Poor	137.25	1.46	+/+	1.10	Hypovascular
3	75	M	1.5	-	Without	Well/Mod.	126.8	1.00	+/-	1.09	Hypovascular
4	65	M	6	+	Without	Well/Mod.	15.67	1.21	+/+	1.05	Hypovascular
5	55	M	7.2	+	With	Poor	10.04	0.74	+/-	1.25	Hypovascular
6	56	M	7.5	+	With	Poor	7.31	0.71	+/-	1.26	Hypovascular
7	76	F	2	-	With	Well/Mod.	5.67	0.91	+/-	1.05	Hypovascular
8	66	M	6.5	+	With	Well/Mod.	3.69	2.15	+/+	1.09	Hypovascular
9	51	M	5.1	-	Without	Well/Mod.	3.2	8.12	+/+	1.25	Hypervascular
10	67	M	10	+	Without	Well/Mod.	2.43	1.37	+/+	1.26	Hypovascular
11	60	M	3	-	With	Well/Mod.	2.06	1.27	+/+	0.83	Hypervascular
12	65	M	2.6	-	Without	Poor	2.01	0.83	+/-	1.18	Hypovascular
13	60	M	4.5	-	With	Well/Mod.	1.66	4.21	+/+	1.19	Hypervascular
14	69	F	5.5	-	Without	Well/Mod.	1.22	2.5	+/+	1.05	Hypervascular
15	65	F	5.5	-	With	Well/Mod.	1.19	1.1	+/+	1.34	Hypovascular
16	66	M	4.5	-	With	Well/Mod.	0	1.02	-/+	1.04	Hypervascular
17	67	M	6.1	+	With	Well/Mod.	0	0.92	-/-	0.94	Hypervascular
18	58	M	4.5	-	With	Well/Mod.	0	0.69	-/-	0.97	Hypervascular
19	62	M	4.5	-	With	Well/Mod.	0	1.54	-/+	1.00	Hypervascular
20	68	M	9.5	+	Without	Well/Mod.	0	1.94	-/+	0.95	Hypervascular
21	65	M	11.4	+	Without	Well/Mod.	0	0.79	-/-	0.94	Hypervascular
22	50	M	17.5	+	With	Well/Mod.	0	1.2	-/+	0.79	Hypervascular
23	73	F	1.5	-	With	Well/Mod.	0	0.6	-/-	0.90	Hypovascular
24	66	M	5	-	With	Poor	0	2.05	-/+	1.00	Hypervascular
25	48	M	12	+	With	Well/Mod.	0	0.77	-/-	0.51	Hypervascular

T/N, tumorous portion/nontumorous portion; HME, human macrophage metalloelastase; VEGF, vascular endothelial growth factor.

\* According to the Classification of Primary Liver Cancer of the Liver Cancer Study Group of Japan.<sup>21</sup>

† Angiostatin was detected by Western blot analysis as previously described,<sup>8</sup> and angiostatin levels were expressed as T/N ratio.

contrast dye was usually infused at 9 mL/sec for 3 seconds through the common hepatic artery. The vascularity of the tumor was evaluated by the degree of tumor-staining intensity into the parenchymal area of the liver at 3.667 seconds after the infusion of contrast dye; this time corresponded to the arterial phase of the angiography. The intensity of tumor staining was determined by detailed study of the angiogram by five surgeons masked to the results of HME and VEGF mRNA expression, and it was classified as hypervascular (strong staining) or hypovascular (moderate or weak staining). There was a consensus of opinion in 14 patients and a 4-to-1 vote in 11 patients.

### Statistical Analysis

The Wilcoxon-Mann-Whitney test compared HME mRNA levels between the hypovascular and hypervascular groups. Fisher's exact probability test was used to evaluate the association of vascularity with HME gene expression. To select an appropriate set of independent variables that significantly affect tumor vascularity in HCC, the clinicopathologic and molecular-biologic variables (see Table 1) were evaluated by stepwise logistic analysis using the like-

lihood ratio criterion. Statistical analyses were performed with JMP 3.0 (SAS Institute, Cary, NC) and with SPSS Professional Statistics 7.5 (SPSS Japan Inc.).  $P < .05$  based on a two-tailed test was considered to indicate statistical significance.

## RESULTS

### Correlation of HME mRNA Expression Level and Tumor Vascularity

In 15 patients, the T/N ratio of HME mRNA expression increased by  $\geq 1.2$ -fold in tumorous tissues compared with nontumorous tissues. In the other 10 patients, no expression of HME gene was detected in the tumorous portion by Northern blot analysis (see Table 1).

Eleven of the 15 patients expressing the HME gene (HME<sup>+</sup>) had hypovascular tumors. In contrast, hypervascular tumors were seen in 9 of the 10 HME-negative patients (Table 2).

The correlation between HME mRNA expression and the intensity of angiographic tumor staining is shown in Figure 1. A close relation was seen between HME mRNA expres-

**Table 2. RELATION OF TUMOR VASCULARITY WITH TUMOR EXPRESSION OF HUMAN MACROPHAGE METALLOELASTASE (HME) mRNA IN HEPATOCELLULAR CARCINOMA**

Variable	Category	HME <sup>+</sup> (n = 15)	HME <sup>-</sup> (n = 10)	P*
Tumor vascularity	Hypovascular	11	1	.004
	Hypervascular	4	9	

\* Fisher's exact probability test.

sion and tumor vascularity in patients with HCC. The median of HME mRNA expression (T/N ratio) was 6.5 (range 0–264.5) in the hypovascular group and 0 (range 0–3.2) in the hypervascular group ( $P = .0004$ , Wilcoxon-Mann-Whitney test).

### Balance Between HME and VEGF mRNA Expression and its Correlation with Tumor Vascularity

A total of 25 patients with HCC were divided, according to their HME and VEGF mRNA expressions (T/N ratios), into the following subgroups: HME ratio > 1.00 and VEGF ratio > 1.00; HME ratio > 1.00 and VEGF ratio ≤ 1.00; HME ratio ≤ 1.00 and VEGF ratio > 1.00; and HME ratio ≤ 1.00 and VEGF ratio ≤ 1.00. For the purpose of discrimination, the following terms—HME<sup>+</sup>/VEGF<sup>+</sup>, HME<sup>+</sup>/VEGF<sup>-</sup>, HME<sup>-</sup>/VEGF<sup>+</sup>, and HME<sup>-</sup>/VEGF<sup>-</sup>—were coined to describe the subgroups respectively (Table 3).

All five patients (100%) from the group HME<sup>-</sup>/VEGF<sup>+</sup> had hypervascular tumors, but in the group HME<sup>+</sup>/VEGF<sup>+</sup> the hypervascular pattern of the tumors decreased to 40% (4/10 patients). In contrast, all patients (100%) in the group HME<sup>+</sup>/VEGF<sup>-</sup> exhibited a hypovascular pattern of staining in their tumors. In the group HME<sup>-</sup>/VEGF<sup>-</sup>, the vascular phenotype of the tumors again shifted to a hypervascular type (80%).

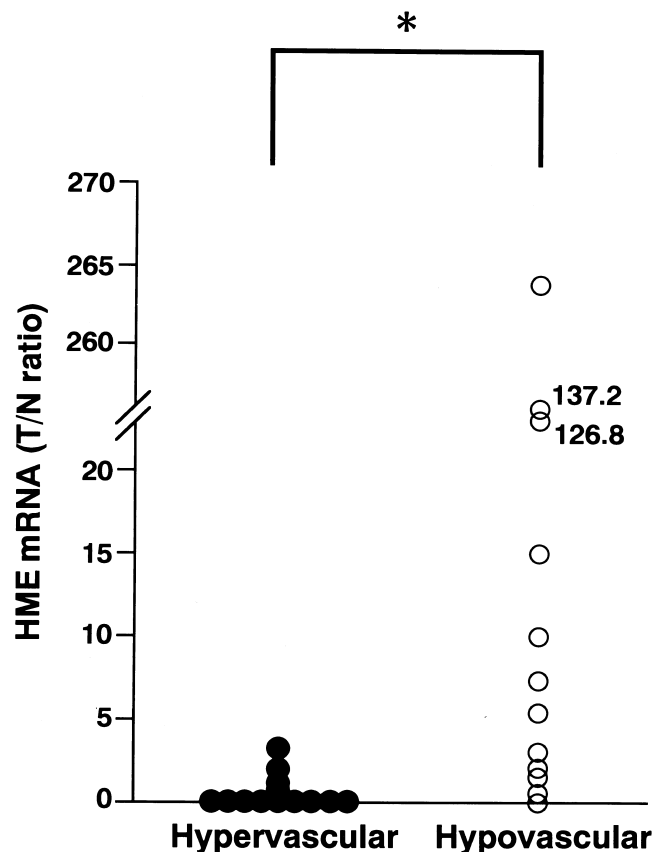
Four patients with high levels of HME mRNA expression (T/N ratio > 1.2) in their tumors also had hypervascular tumors. However, in three of them, the VEGF ratio increased by more than twofold compared with the HME ratio (patients 9, 13, and 14). In the fourth patient (patient 11), the level of angiostatin generation by the tumor was lower than in its corresponding nontumorous tissue (angiostatin T/N ratio = 0.83) (see Table 1).

### Results of Stepwise Logistic Analysis

HME expression and VEGF mRNA expression were selected as two independent variables that significantly affect the vascularity of HCC (likelihood ratio chi-square = 15.2,  $P = .0001$ ; likelihood ratio chi-square = 5.4,  $P = .02$ , respectively).

### Angiography Findings

To illustrate the relation of HME and VEGF mRNA expression with hepatic angiographic findings, four representative cases were chosen. Figure 2 shows the Northern blot results for HME and VEGF mRNA expression; Figure 3 shows the angiograms. Patient 21, who had HME and VEGF T/N ratios of 0 and 0.79 respectively, with no generation of angiostatin (T/N ratio 0.79), showed strong angiographic staining (see Fig. 3A). In patient 22, the HME and VEGF T/N ratios were 0 and 1.2 respectively, also with no generation of angiostatin (T/N ratio 0.79). This patient showed stronger angiographic staining than patient 21 (see Fig. 3B). Conversely, patient 7, who showed HME mRNA expression (T/N ratio 5.67) with a very low expression of VEGF (T/N ratio 0.91) and angiostatin generation (T/N ratio 1.05), showed weak tumor staining in the angiogram (see Fig. 3C). Patient 8, who expressed a relatively high level of VEGF mRNA (T/N ratio 2.15), but with a higher expression of HME gene (T/N ratio 3.69) and angiostatin



**Figure 1.** Scattergram showing the correlation between the intensity of angiographic tumor staining and human macrophage metalloelastase (HME) mRNA expression levels. The y axis represents HME mRNA in the tumorous portion/HME mRNA in the nontumorous portion (T/N ratio). The HME T/N ratio varied from 0 to 264.5 (median 6.5) in the patients with hypovascular tumors (n = 12) and from 0 to 3.2 (median 0) in the patients with hypervascular tumors (n = 13). (\* $P = .0004$  by Wilcoxon-Mann-Whitney test).



**Table 3. RELATION OF TUMOR VASCULARITY WITH TUMOR EXPRESSION OF HUMAN MACROPHAGE METALLOELASTASE (HME) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN HEPATOCELLULAR CARCINOMA**

Variable	Category	HME/VEGF mRNA Expression			
		+/+ (n = 10)	+/- (n = 5)	-/+ (n = 5)	-/- (n = 5)
Tumor vascularity	Hypovascular	6	5	0	1
	Hypervascular	4	0	5	4

generation (T/N ratio 1.09), showed weak tumor staining as well (see Fig. 3D).

### Association Between HME or VEGF mRNA Expression and Histopathologic Findings

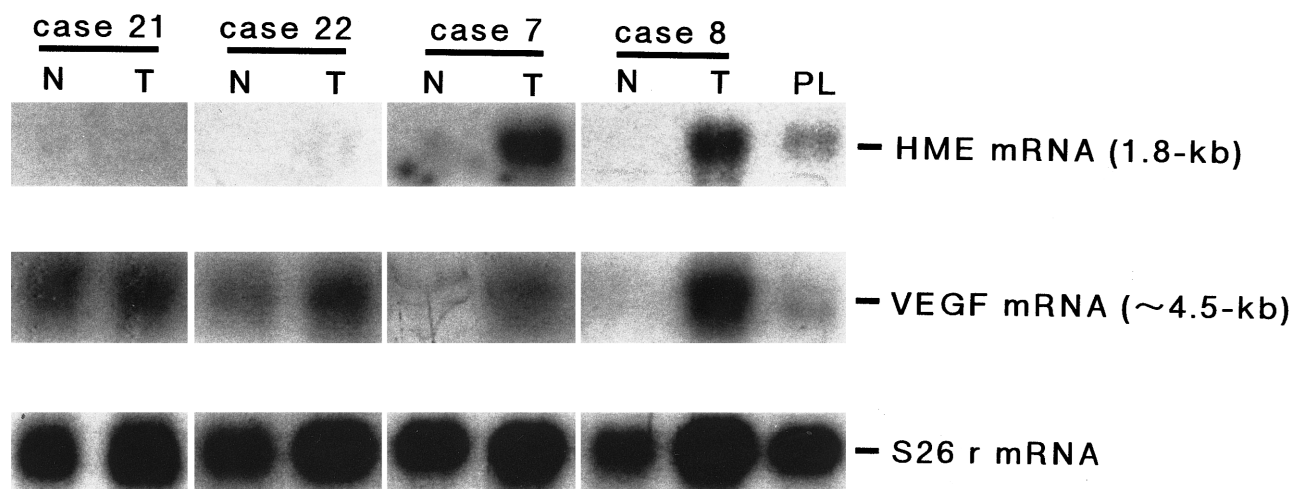
Table 1 shows the clinicopathologic features in each patient. No significant association of HME or VEGF mRNA expression levels with the tumor size, portal vein invasion, pathologic grade of anaplasia, or cirrhosis was found. Moreover, these clinicopathologic features did not show significant association with tumor vascularity (data not shown). Thus, the clinicopathologic backgrounds were uniform between the four HME/VEGF groups.

### DISCUSSION

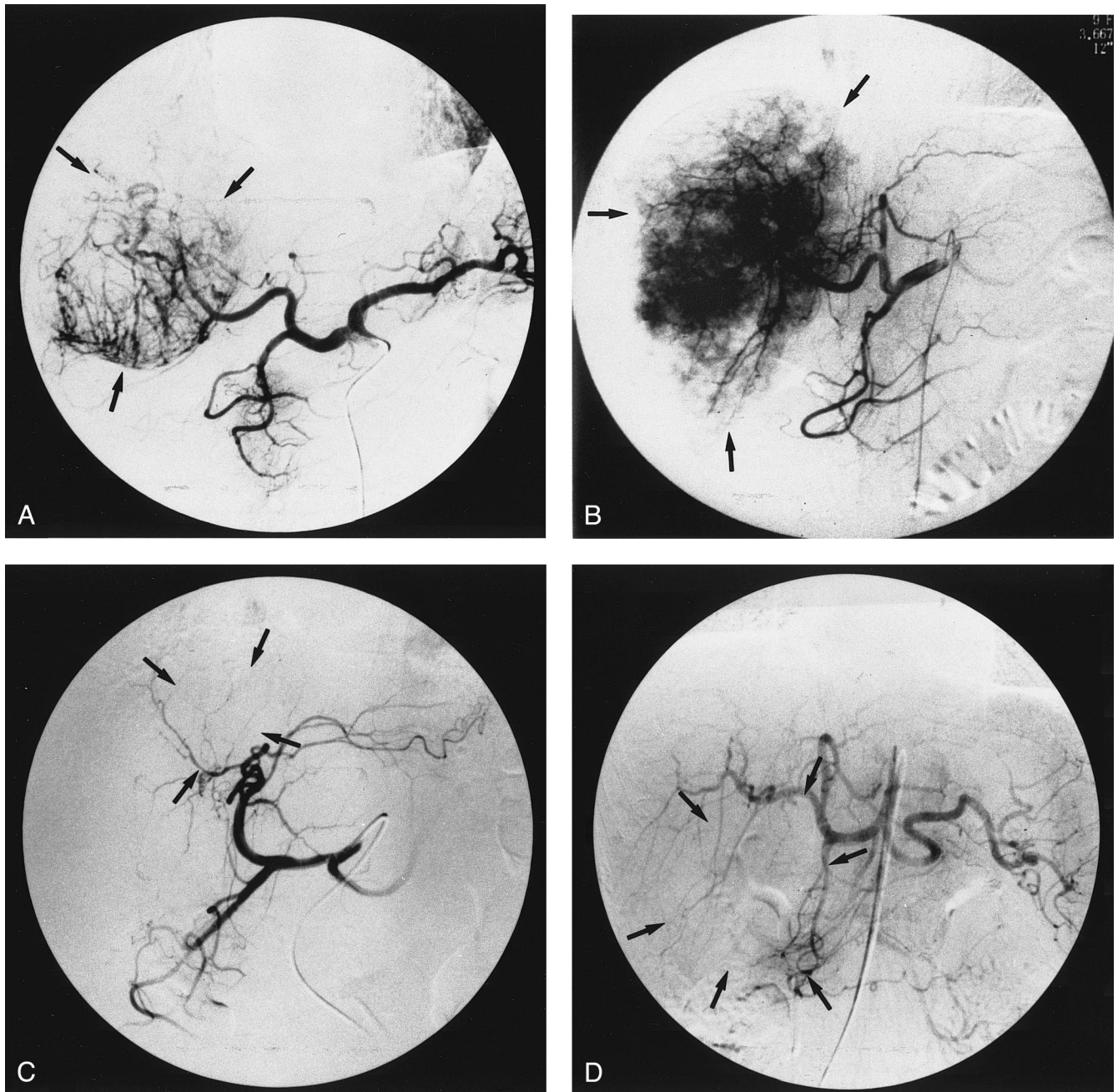
HCCs are commonly regarded as hypervascular tumors, although their vascularity ranges from a relatively hypovascular to a hypervascular pattern.<sup>20,22</sup> Previous reports have demonstrated that angiogenesis is a prerequisite for the development of HCC from precancerous foci.<sup>23-26</sup> Thus,

more studies regarding the mechanism of angiogenesis in HCC are important because a better understanding of the molecular network involved in new vessel formation may contribute to the establishment of novel therapeutics (as well as biologic interest).

In our previous study, we reported that HME, a type of MMP, can be expressed in some HCCs (62.5%), and that its expression is strongly associated with the production of angiostatin, an endogenous inhibitor of angiogenesis.<sup>8</sup> The present study provides clinical evidence that the angiogenesis of HCC mainly depends on the expression of the HME gene, because 73% of the patients whose tumors expressed HME gene showed moderate or weak staining in hepatic angiograms, and 90% of the patients whose tumors did not express HME gene showed strong staining, indicating that the expression of HME effectively inhibits angiogenesis in HCC ( $P = .004$ ; see Table 2). In fact, the Wilcoxon-Mann-Whitney test revealed a median HME T/N ratio of 6.5 (range 0-264.5) in the hypovascular group, compared with a median HME T/N ratio of 0 (range 0-3.2) in the hypervascular group ( $P = .0004$ ).



**Figure 2.** Northern blot analysis for human macrophage metalloelastase (HME) mRNA expression (upper panel) and vascular endothelial growth factor (VEGF) mRNA expression (middle panel) in tumorous and nontumorous liver tissues from four patients with hepatocellular carcinoma (patients 21, 22, 7, and 8). Five micrograms of polyA<sup>+</sup> RNA from each human tissue were used. Hybridization was performed with HME and VEGF<sub>165</sub> cDNA as described in text. The 1.8-kb band and ~4.5-kb band, corresponding to the HME and VEGF transcripts respectively, were detected. S26 r mRNA expression is shown as the corresponding internal control in the lower panel. N, nontumorous portion; T, tumorous portion; PL, human placenta.



**Figure 3.** Hepatic angiograms through the celiac trunk with the catheter tip positioned in the common hepatic artery, showing the degree of tumor-staining intensity in patients 7, 8, 21, and 22. (A) Angiogram of patient 21, exhibiting strong tumor staining; human macrophage metalloelastase (HME) and vascular endothelial growth factor (VEGF) tumorous/nontumorous (T/N) ratios were 0 and 0.79, respectively. (B) Angiogram of patient 22, showing the strongest tumor staining; HME and VEGF T/N ratios were 0 and 1.2, respectively. (C) Angiogram of patient 7 and (D) angiogram of patient 8, both showing weak tumor staining; HME and VEGF T/N ratios were 5.67 and 0.91 respectively in patient 7 and 3.69 and 2.15 respectively in patient 8. Tumor location is denoted by the arrows.

The correlation of both HME and VEGF gene expression with tumor vascularity confirmed that both factors are significantly associated with the vascularity of HCC; however, it seems to be mainly determined by HME gene expression. In addition, the hypovascular phenotype shown by HME-expressing HCC shifted toward the hypervascular phenotype only in

two situations: first, when the VEGF T/N ratio increased by more than twofold over HME mRNA expression levels (patients 9, 13, and 14; see Table 1), and second, when the tumors did not produce angiostatin despite HME gene expression (patient 11). Moreover, in the patients who did not express the HME gene and therefore had hypervascular tumors, a VEGF



mRNA expression level of 1.2 or more was associated with stronger vascular staining in the angiograms (as shown in Fig. 3B, patient 22) than in patients whose tumors showed low levels (<1.00) of VEGF mRNA expression (see Fig. 3A, patient 21). Thus, although overproduction of angiogenic stimulators is necessary for neovascularization in HCC, they are not sufficient for the angiogenic switch.

In our series, four patients with HCC whose tumors did not express HME and in addition showed low levels of VEGF mRNA expression had hypervascular tumors (patients 17, 18, 21, and 25). The Northern blot and angiogram results of one of these patients (patient 21) are shown in Figures 2 and 3A, respectively. This might be explained, to a lesser extent, by the participation of other angiogenic factors, such as scatter factor (hepatocyte growth factor), basic fibroblast growth factor, platelet derived-endothelial cell growth factor, or others.<sup>3</sup>

This study provides insights into the molecular basis of angiogenesis in HCC. We confirmed that HME-expressing HCCs will develop tumors with a relatively hypovascular phenotype and that it might be turned into a hypervascular phenotype, depending on the levels of VEGF mRNA expression. Thus, it is not difficult to speculate that the hypovascular phenotype shown by HME-expressing HCCs might be due to the presence and activity of angiostatin generated by these tumors through HME (see Table 1).

In conclusion, HME mRNA expression is significantly associated with hypovascular tumors. We demonstrated that the vascularity of HCC does not depend on a single factor, but on a net balance between HME and VEGF. Finally, considering that the development of HCC is strongly dependent on neovascularization, the molecular mechanism of angiogenesis demonstrated here provides bases for the establishment of a novel therapeutic and preventive strategy against HCC. Thus, further studies are needed focusing on the blockade of angiogenesis by enhancing HME gene expression by specific gene transfer or through the suppression of VEGF gene expression by using an antisense approach, such as VEGF gene targeting or antibodies. Then, combinations of conventional chemotherapy and antiangiogenic gene therapy may improve the survival rate after surgery.

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