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## Abstract

The activity of serum type IV collagen-degrading enzyme was measured in 18 patients with hepatocellular carcinoma (HCC). The enzyme activity was significantly higher, in HCC patients with a tumor thrombus in the portal vein than in healthy controls, liver cirrhosis patients and HCC patients without a tumor thrombus. Moreover, the activity in HCC patients with lung metastasis tended to be higher than that in HCC patients without lung metastasis. The activity of serum type IV collagen-degrading enzyme did not correlate with tumor size, serum alpha-fetoprotein level, or macroscopic classification of tumor growth. These results suggest that the activity of serum type IV collagen-degrading enzyme represents the metastatic potential or the ongoing metastatic activity of HCC. The enzyme is a useful serum marker of metastasis from HCC.

**KEYWORDS:** type IV collagen-degrading enzyme, hepatocellular carcinoma, vascular invasion, metastasis, collagenase

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## Serum Type IV Collagen-Degrading Enzyme in Hepatocellular Carcinoma with Metastasis

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The activity of serum type IV collagen-degrading enzyme was measured in 18 patients with hepatocellular carcinoma (HCC). The enzyme activity was significantly higher, in HCC patients with a tumor thrombus in the portal vein than in healthy controls, liver cirrhosis patients and HCC patients without a tumor thrombus. Moreover, the activity in HCC patients with lung metastasis tended to be higher than that in HCC patients without lung metastasis. The activity of serum type IV collagen-degrading enzyme did not correlate with tumor size, serum  $\alpha$ -fetoprotein level, or macroscopic classification of tumor growth. These results suggest that the activity of serum type IV collagen-degrading enzyme represents the metastatic potential or the ongoing metastatic activity of HCC. The enzyme is a useful serum marker of metastasis from HCC.

*Key words* : type IV collagen-degrading enzyme, hepatocellular carcinoma, vascular invasion, metastasis, collagenase

Hepatocellular carcinoma (HCC) invades the portal vein to metastasize intrahepatically and the hepatic vein to metastasize extrahepatically (1). It is clinically important to know the metastatic potential and ongoing metastatic activity of HCC. However, no simple and useful marker of tumor metastasis has been reported yet. The tumor cells must invade the vascular wall or lymphatic channels and migrate out of the circulation to colonize various organs. In these processes, the basement membranes of blood vessels must be degraded by tumor cells (2). Type IV collagen is one of the major components of basement membrane (3), and

is resistant to digestion by usual proteinases, but is specifically degraded by type IV collagen-degrading enzyme (4). Therefore, type IV collagen-degrading enzyme is thought to play an important role in tumor metastasis (5, 6). As many types of inhibitors of collagenase and other proteinases such as  $\alpha_2$ -macroglobulin and  $\beta_1$ -anticollagenase exist in the circulating blood, the determination of the activity of type IV collagen-degrading enzyme in serum has been unsuccessful. Recently, the serum enzyme activity was successfully determined in our laboratory (7).

In the present study, type IV collagen-degrading enzyme activity was determined in serum obtained from patients with HCC

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with and without a tumor thrombus in the portal vein or lung metastasis. The clinical importance of this enzyme, especially as a serum marker of HCC metastasis, is discussed.

## Materials and Methods

**Patients.** Eighteen male patients with HCC complicated with liver cirrhosis (HCC-LC group; average age of 58 years, range 48-75) were examined (Table 1). A tumor thrombus in the portal vein, which was diagnosed by real-time ultrasonography (EUB-400; Hitachi Medico Co. Ltd., Tokyo) and/or celiac angiography, was detected in 11 out of the 18 HCC patients. The macroscopic classification of tumor growth was done using ultrasonography and angiography according to the classification of the Liver Cancer Study Group of Japan (8). Tumor diameter was measured

on ultrasonograms and angiograms. Fifteen male patients with liver cirrhosis (LC group; average age of 55 years, range 40-72), who were confirmed to be free of HCC by ultrasonography and angiography, and 19 healthy male subjects (control group; average age of 59 years, range 43-82) were also examined as controls. Serum was separated from venous blood obtained in the early morning.

**Preparation of substrate.** Type IV collagen was purified from human placentas according to the method of Sage *et al.* (9), and radiolabeled with [ $^{14}\text{C}$ ]acetic anhydride (New England Nuclear, Boston, MA) by a method modified from that of Cawston *et al.* (10).

**Enzyme activation.** Sixty  $\mu\text{l}$  of serum were preincubated with 1 mM 4-aminophenylmercuric acetate (APMA; Wako Pure Chemical Industries Ltd., Osaka) and 435  $\mu\text{g}$  trypsin (Sigma Chemical Co., St. Louis, MO), as described previously (7).

**Assay for type IV collagen-degrading enzyme**

**Table 1** Clinical features of 18 patients with hepatocellular carcinoma

Case	Age	HBs Ag/Ab	Alcohol drinking <sup>a</sup>	AFP (ng/ml)	Macroscopic classification	Tumor size <sup>b</sup> (mm)	No. of tumor nodules <sup>c</sup>	Tumor thrombus in the portal vein	Metastasis to the lung
1	72	+/-	-	10	Nodular	32	3	-	-
2	54	-/-	+	600	Nodular	40	4	-	-
3	54	-/-	-	1770	Diffuse	N.M. <sup>d</sup>	5 $\leq$	-	-
4	68	-/+	-	318	Nodular	45	1	-	-
5	55	-/+	+	4	Nodular	40	2	-	-
6	63	-/-	+	128	Nodular	43	4	-	-
7	75	-/-	-	69	Nodular	20	3	-	-
8	53	+/-	-	71800	Massive	145	1	+	-
9	58	+/-	-	3150	Massive	63	5 $\leq$	+	-
10	62	+/-	+	27100	Diffuse	N.M.	5 $\leq$	+	-
11	49	+/-	-	2129	Massive	130	5 $\leq$	+	-
12	48	-/-	-	79	Massive	120	1	+	-
13	62	-/-	+	456	Nodular	41	2	+	-
14	49	-/-	-	6660	Nodular	41	5 $\leq$	+	-
15	60	-/-	-	157	Nodular	55	5 $\leq$	+	-
16	56	+/-	-	2103	Diffuse	N.M.	5 $\leq$	+	+
17	50	-/+	+	365	Diffuse	N.M.	5 $\leq$	+	+
18	54	-/-	-	2	Diffuse	N.M.	5 $\leq$	+	+

*a*: +, More than 80 g ethanol daily for over 5 years.

*b*: The maximum diameter of the main tumor.

*c*: The number of tumor nodules was determined by macroscopically counting the nodules on the ultrasonograms and angiograms.

*d*: Not measured because of diffuse type.

Abbreviations: HBs Ag/Ab, Surface antigen/antibody of hepatitis B virus; AFP,  $\alpha$ -fetoprotein.

activity. Sixty-five  $\mu\text{g}$  of  $[1\text{-}^{14}\text{C}]$ -labeled type IV collagen and the serum preincubated prior to assay were incubated at  $37^\circ\text{C}$  for 5 h in 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM  $\text{CaCl}_2$ , 200 mM NaCl and 1 mM diisopropyl fluorophosphate (DFP; Sigma), as described previously (7). Each assay was done in duplicate. The reaction mixture with 10 mM ethylenediaminetetraacetic acid (EDTA) was simultaneously run as the control. One unit of type IV collagen-degrading enzyme activity was defined as 1  $\mu\text{g}$  of collagen degraded at  $37^\circ\text{C}$  per min.

**Statistical analysis.** The results obtained were expressed as the mean  $\pm$  the standard deviation (SD) of the mean. Group means were compared by multiple comparison following analysis of variance. Correlations were calculated by linear regression analysis using the method of least squares.

## Results

Serum type IV collagen-degrading enzyme activity in the LC group was not higher than that in the control group (Fig. 1). The enzyme activity in the HCC-LC group without a tumor thrombus in the portal vein was not higher than that in the LC group. However, the activity in the HCC-LC group with a tumor thrombus was higher than that in the LC group and that in the HCC-LC group without a tumor thrombus. Moreover, the activity in the HCC patients with lung metastasis tended to be higher than that in the HCC patients without lung metastasis (see Fig. 1). There was no relationship between the enzyme activities and the mac-

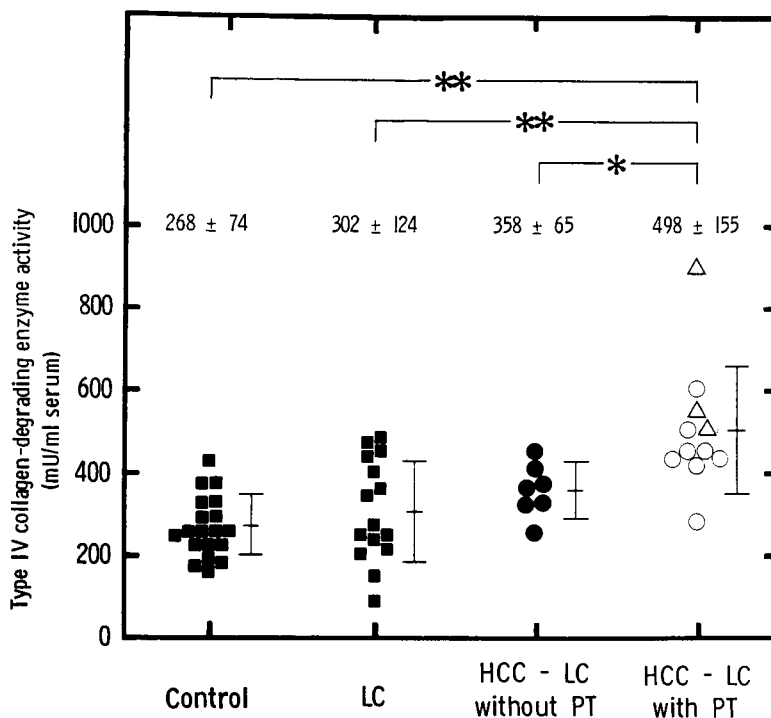


Fig. 1 The activity of serum type IV collagen-degrading enzyme in patients with hepatocellular carcinoma and liver cirrhosis (HCC-LC) with (○) and without (●) a tumor thrombus in the portal vein (PT), in patients with liver cirrhosis (■)(LC), and in healthy subjects (■)(Control).  $\Delta$ : Patients with lung metastasis. Upper and lower horizontal lines indicate SD of the mean, and middle horizontal lines indicate the mean.

\*:  $p < 0.05$ . \*\*:  $p < 0.001$ .

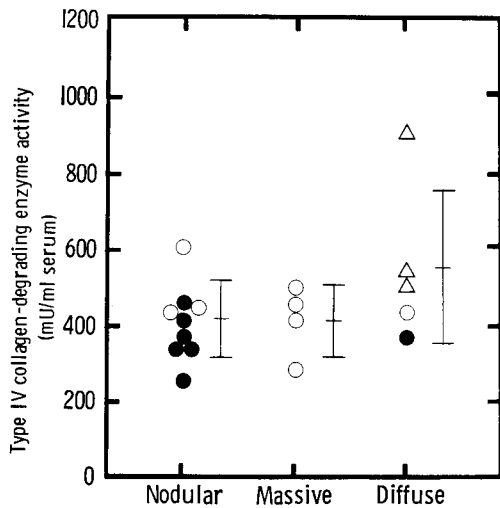


Fig. 2 The activity of serum type IV collagen-degrading enzyme in patients with hepatocellular carcinoma in relation to the macroscopic type of tumor growth. See the legend for Fig. 1 for more details.

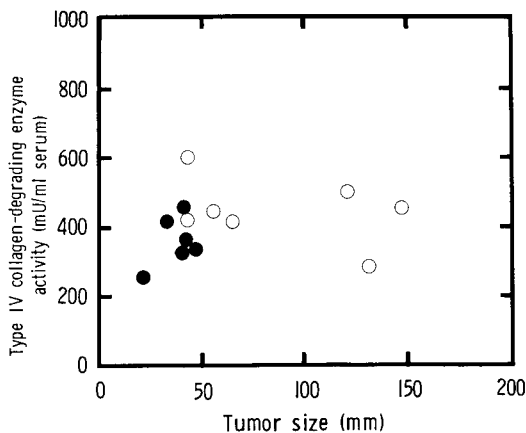


Fig. 3 The activity of serum type IV collagen-degrading enzyme in relation to the maximum diameter of the main tumor in hepatocellular carcinoma patients. Five patients (Cases 3, 10, 16, 17 and 18) were excluded because the carcinoma was of the diffuse type. See the legend for Fig. 1 for more details.

rosopic type of tumor growth (Fig. 2), tumor size (Fig. 3), or number of tumor nodules (Fig. 4). The enzyme activity did not correlate with the serum  $\alpha$ -fetoprotein level,

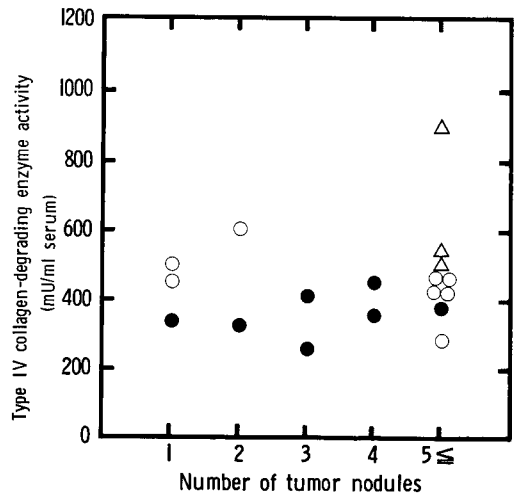


Fig. 4 The activity of serum type IV collagen-degrading enzyme in hepatocellular carcinoma in relation to the number of tumor nodules. The number of tumor nodules was determined by macroscopically counting the nodules on ultrasonograms and angiograms. See the legend for Fig. 1 for more details.

bilirubin concentration or activities of aspartate aminotransferase and alanine aminotransferase.

## Discussion

The activity of serum type IV collagen-degrading enzyme was high in the HCC-LC group with a tumor thrombus in the portal vein. Intrahepatic metastasis of HCC occurs via the portal vein (1). The first step of intrahepatic metastasis is the entering of HCC cells into the portal vein. After migration, HCC cells have to pass again through the wall of the portal vein to invade the liver tissue. In these processes, the basement membrane of the portal vein acts as a barrier to the passage of HCC cells. For metastasis to occur, it is necessary that HCC cells locally destroy the structure of the basement membrane, which is composed

mainly of type IV collagen (3). Therefore, it is likely that HCC cells secrete type IV collagen-degrading enzyme and invade the basement membrane resulting in a tumor thrombus.

On the other hand, HCC forms extrahepatic or remote metastasis mainly via the hepatic vein (1). In the present investigation, 3 HCC patients had lung metastasis, and all these patients had high activities of serum type IV collagen-degrading enzyme. Therefore, the enzyme activity is elevated not only in intrahepatic metastasis but also in extrahepatic or remote metastasis.

It has been reported from our laboratory that the activity of liver type IV collagen-degrading enzyme was high in the cancer border of HCC autopsy materials (11). The highest activity of the liver enzyme was revealed in HCC patients with marked metastasis to the lung (12). Moreover, the activity of type IV collagen-degrading enzyme was demonstrated to correlate with the metastatic potential of murine sarcoma and melanoma cells (5). Thus, the increased activity of serum type IV collagen-degrading enzyme, which is probably produced by HCC cells, might reflect metastatic potential or ongoing metastatic activity. The activity of serum type IV collagen-degrading enzyme did not correlate with tumor size, nor was there any relationship between this enzyme and the type of tumor growth. As HCC develops in cirrhotic liver, HCC cells are surrounded by abundant connective tissue. Other proteinases, such as interstitial collagenase, also participate in expansive or contiguous invasion of HCC, more than type IV collagen-degrading enzyme does. No clear relationship existed between this enzyme activity and the number of tumor nodules.

Not much is known at present about the metabolism of type IV collagen-degrading enzyme. The change in the blood flow due to the formation of a tumor thrombus in the

portal vein may partly delay the turnover of type IV collagen-degrading enzyme. However, it is unlikely that changes in the blood flow increase this enzyme activity in the serum, because there was no relationship between the size of the tumor thrombus and the enzyme activity.

Laminin, which is also one of the major components of basement membrane (13), participates in tumor metastasis as a protein which attaches between the tumor cell and basement membrane (14). Moreover, laminin increases the release of type IV collagen-degrading enzyme from malignant cells (15). However, as the serum laminin level is high in patients with liver cirrhosis and showed no difference between patients with liver cirrhosis and patients with HCC and liver cirrhosis (16), the serum laminin level is not a useful marker of HCC metastasis.

It is very difficult to absolutely cure HCC patients with liver cirrhosis, because of poor liver function, formation of intrahepatic metastasis and so on. At present, there are various treatments for HCC, such as surgery, transcatheter arterial embolization (TAE), chemotherapy, immunotherapy and radiation therapy. However, it is often difficult to choose an appropriate treatment and to evaluate the effectiveness of the adopted therapy. Therefore, it is very important to know the metastatic potential and ongoing metastatic activity of individual HCC patients. Measuring the activity of serum type IV collagen-degrading enzyme in HCC patients, as a serum marker of metastasis, may be useful in choosing the appropriate treatment.

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