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Short Communication

Cellular Distribution of 92-kd Type IV Collagenase/Gelatinase B in Human Hepatocellular Carcinoma

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To examine the possible involvement of gelatinase B in human hepatocellular carcinoma (HCC), cellular localization of transcripts and protein of gelatinase B were studied by using* in situ *hybridization and immunohistochemistry. Transcripts for gelatinase B were observed in tumor cells in 22 cases of 27 HCCs and also in dysplastic nodules. However, there was no significant difference in the expression among histological grades of HCC. The expression was mostly homogeneous, but the intensity varied with the nodules. Of 13 cases with capsular invasion, 12 expressed gelatinase B, whereas 10 of 14 without capsular invasion did ($P < 0.05$). Gelatinase B transcripts were commonly observed in the sinusoidal cells of the hepatic lobules, in mesenchymal cells both in fibrous capsules and around the necrosis, and also in some undefined cells of the portal tracts of noncancerous liver. Localization of gelatinase B protein was mostly similar to but sometimes different from that of the transcripts in cancer nodules. In conclusion, the expression of gelatinase B appears to be an important characteristic of malignant transformation of hepatocytes. The findings suggest that gelatinase B synthesized by cancer cells plays an important role in the growth and invasion of HCC by degrading surrounding extracellular matrices. (Am J Pathol 1996, 149:1803–1811)

Hepatocellular carcinoma (HCC) is one of the worst prognostic cancers, and the mortality in Japan has been more than 27,000 per year.[1] Recurrence and intrahepatic metastasis of HCC are frequently observed and are the most difficult problems in clinical practice.[2] Most HCCs occur in chronic liver diseases, such as chronic viral hepatitis and liver cirrhosis in which abundant extracellular matrix exists. Furthermore, HCC is commonly surrounded by fibrous capsule[3] even in the early stages. Therefore, breakdown of capsule and surrounding extracellular matrix appears to be essential for the growth of HCC, particularly for the process of invasion and metastasis.

Gelatinase B has been reported to degrade type IV collagen and also gelatin (denatured collagens), type V, VII, and X collagens, casein, and elastin.[4–8] Type IV collagen is the major collagen component of basement membrane. Invasion of tumor cells into the basement membrane is the initial step of cancer metastasis. The degradation of type IV collagen needs to be initiated by specific enzymes such as 92-kd gelatinase/gelatinase B (MMP-9), 72-kd gelatinase/gelatinase A (MMP-2), and stromelysins. The triple helix of native type I and III collagens is normally cleaved by interstitial collagenases but not by gelatinases. However, it has been recently reported that gelatinase B also degrades the $\alpha 2$ chain of type I and the $\alpha 1$ chain of type III collagens.[9,10] Type I and III collagens are the major collagen components of the fibrous capsule of HCC as well as of fibrotic liver.[11,12] Therefore, gelatinase B may play an important role in the degradation of connective tis-

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Table 1. *Patient Data*

Patient	Age (years)	Sex	Virus	Liver disease	Size (mm)	Differentiation grade	Capsule formation	Capsular invasion
1	57	M	C	LC	18 × 11	Well	−	−
2	49	M	C	CH	14 × 13	Well	+	+
3	64	M	C	CH	15 × 15	Well	+	+
4	67	M	C	LC	45 × 30	Well	+	+
5	71	F	C	LC	19 × 17	Well	+	−
6	70	F	C	LC	20 × 17	Well	−	−
7	67	M	−	CH	35 × 20	Well	+	−
8	45	M	B,C	CH	20 × 16	Well	+	−
9	75	M	−	CH	19 × 17	Mod	+	+
10	65	M	C	LC	22 × 20	Mod	+	+
11	44	M	B	CH	27 × 27	Mod	+	+
12	67	M	B	CH	31 × 25	Mod	+	+
13	62	M	B*	CH	40 × 35	Mod	+	+
14	49	M	B	CH	50 × 47	Mod	+	+
15	65	M	B,C	LC	50 × 47	Mod	+	+
16	76	F	−	CH	58 × 38	Mod	+	+
17	61	M	B	LC	18 × 16	Mod	+	−
18	55	M	C	LC	20 × 20	Mod	+	−
19	77	M	C	LC	20 × 15	Mod	+	−
20	60	M	C	CH	25 × 18	Mod	+	−
21	58	F	B,C	LC	33 × 30	Mod	+	−
22	73	M	B	CH	34 × 29	Mod	+	−
23	62	F	C	CH	50 × 45	Mod	+	−
24	60	F	C	LC	100 × 70	Poor	+	+
25	63	M	C	LC	100 × 87	Poor	+	+
26	74	M	C	LC	23 × 16	Poor	+	−
27	60	M	C	LC	30 × 30	Poor	+	−

M, male; F, female; C, hepatitis C virus antibody positive; B, hepatitis B surface antigen positive; CH, chronic hepatitis; LC, liver cirrhosis; Mod, moderately.
*Hepatitis C virus antibody; unknown.

sue barriers during the growth, invasion, and metastasis of HCCs and may determine the invasive potential of HCCs in fibrotic liver.

As the cellular source, neutrophils and macrophages have been reported to release gelatinase B.[5,6] Transcripts for gelatinase B in colon cancer[13] and ovarian cancer[14] have been found only in macrophages around cancer nodules but not in cancer cells. However, other studies described that the gelatinase B gene was chiefly expressed in cancer cells of skin,[15] lung,[16] salivary gland,[17] breast,[18] pancreas,[19] and cervix.[20]

In this study, we characterized the potential involvement of gelatinase B in the growth and invasion of human HCCs. We examined the expression of gelatinase B in HCC nodules and the cell types that are responsible for the synthesis of gelatinase B. Localization of both transcripts and protein of gelatinase B in human HCC were studied by using *in situ* hybridization and immunohistochemistry.

Materials and Methods

Patients

Twenty-seven patients with HCC (aged 44 to 77 years) were studied. Table 1 shows the clinical diagnosis and patient data. Informed consent was obtained from each patient.

Liver Samples

As soon as the tissues were obtained, a slice of each sample was fixed in 10% neutral formalin and embedded in paraffin. The other part of the sample was separated into the cancer and its noncancerous counterpart, and the tissues were frozen and stored at −80°C until zymogram analysis. Four-micron sections were prepared and were put onto silanized slides (Dako, Tokyo, Japan). The serial sections were used for *in situ* hybridization, immunohistochemistry, and hematoxylin and eosin (H&E) staining for histological diagnosis.

Immunohistochemistry

For immunohistochemistry, the indirect immunoperoxidase method was used. Briefly, tissue sections were deparaffinized by 100% xylene and then hydrated with a graded series of ethanol. The endogenous peroxidase was eliminated by incubation in 3% hydrogen peroxide for 30 minutes, and nonspecific binding of IgG to tissue protein was blocked by incubation with

10% normal rabbit serum for 1 hour. The sections were then reacted with monoclonal anti-human gelatinase B antibody raised in mouse (Fuji Chemical Co., Toyama, Japan) at a 1:250 dilution at 4°C overnight. Biotinylated anti-mouse antibody was used as the secondary antibody followed by peroxidase-streptavidin complex (Histofine, Nichirei, Japan). The slides were rinsed three times with phosphate-buffered saline (PBS, pH 7) after each step. The peroxidase activity was revealed by 0.05% 3,3′-diaminobenzidine tetrahydrochroride (Histofine), and hematoxylin was used for nuclear staining. For each experiment, negative controls omitting either primary or secondary antibodies were included to examine nonspecific staining.

Probes

The plasmid containing a 335-bp fragment for progelatinase B cDNA (nucleotides 1311 to 1645) was a generous gift from Dr. A. Mackay (National Cancer Institute, Bethesda, MD). The cDNA was amplified by standard polymerase chain reaction and subsequently subcloned in pGEM3z (Promega, Madison, WI). The amplified clone was verified by analyzing the nucleotide sequence. The antisense and sense (as a negative control) riboprobes were made by *in vitro* run-off transcription after linearization of the plasmid with *Hin*dIII or *Eco*RI, respectively. Single-stranded riboprobes were transcribed from 1 μg of DNA template with 3.5 mmol/L digoxigenin-labeled UTP, 2 μl of 10X nucleoside triphosphate (<10 mmol/L each of ATP, CTP, and GTP and 6.5 mmol/L UTP) labeling mixture, and with 40 U of T7 or SP6 RNA polymerases (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 1 hour. The riboprobes were confirmed by running RNA gels, and the concentration was measured by a spectrophotometer.

In Situ *Hybridization*

Tissue sections were first deparaffinized, hydrated, and incubated in 0.2 N HCl with 250 μg/ml pepsin for 15 minutes at 37°C. After being washed with 0.3% Triton X-100 in PBS, the sections were treated with 20 μg/ml proteinase K (Boehringer Mannheim) in 0.1 mol/L Tris/HCl (pH 8) and 50 mmol/L EDTA for 30 minutes at 37°C and fixed with freshly prepared 4% paraformaldehyde in PBS for 5 minutes. The slides were rinsed in 0.2% glycine and 0.2 mol/L Tris (pH 7.4) for 10 minutes and acetylated in 0.25% acetic anhydride (freshly prepared) in 0.1 mol/L triethanolamine for 10 minutes. Sections were prehybridized in 50% formamide and 2X standard saline citrate (SSC) for 2 hours at 42°C. Hybridization was performed in 80 μl of hybridization buffer with 2 μg/ml denatured riboprobe in a moist chamber for 12 hours at 42°C. Hybridization buffer contained 0.01 mol/L Tris/HCl (pH 7.5), 12.5% Denhardt's solution, 2X SSC, 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and 250 μg/ml herring sperm DNA. After being rinsed in 2X SSC for 5 minutes at 42°C, the slides were washed twice with 0.1X SSC for 20 minutes at 42°C. After immersion in 2X SSC, the tissues were treated with RNAse A (100 μg/ml diluted with 2X SSC) for 30 minutes at 37°C. After the final wash for *in situ* hybridization with 2X SSC for 10 minutes at room temperature, sections were then forwarded to the immunological reaction for revealing digoxigenin. This reaction was allowed to proceed by following the manufacturer's protocol (Boehringer Mannheim) at room temperature unless otherwise described. After immersion with buffer 1 (100 mmol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 5 minutes at room temperature, the slides were incubated in buffer 2 (buffer 1 containing 1% blocking reagent) for 30 minutes. Then 80 μl of alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments diluted at 1:500 in buffer 2 was applied and incubated for 3 hours at 4°C. Slides were washed twice in buffer 1 containing 0.3% Tween 20 for 20 minutes and rinsed in buffer 3 (100 mmol/L NaCl, 20 mmol/L $MgCl_2$, 100 mmol/L Tris/HCl, pH 9.5). Bound antibody was detected by a standard immunoalkaline phosphatase reaction, using 200 mg/ml nitroblue tetrazolium chloride/250 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in buffer 3 as substrate.

Gelatin Zymogram

Gelatinolytic activity was analyzed by gelatin zymogram according to the previous report.[21] Briefly, liver samples were homogenized in collagenase buffer containing 50 mmol/L Tris/HCl, 0.2 mol/L NaCl, 10 mM $CaCl_2$, pH 7.4, and were then centrifuged at 10,000 × *g*. Ten micrograms (protein concentration) of the supernatant was applied to the SDS gel containing 0.1% gelatin. After electrophoresis and wash of the gel, the gel was incubated at 37°C for 16 hours and stained with 0.1% Amido Black.

Statistical Analysis

The χ^2 test was used for statistical analysis.

Results

Cellular localization of transcripts for gelatinase B was examined in 27 human HCCs by means of *in situ* hybridization. Gene transcripts for gelatinase B were observed in cancer cells of 22 cases (81%) of 27 HCCs. Mesenchymal cells in and out of cancer nodules also expressed gelatinase B (Figure 1A). In each cancer nodule, the distribution of the transcripts was homogeneous, but signal intensity varied among different nodules (Figure 1B). Expression of gelatinase B was observed in various differentiation grades of HCC. Transcripts for gelatinase B were found in 6 of 7 (86%) well differentiated HCCs, 13 of 15 (86%) moderately differentiated HCCs, and 3 of 4 (75%) poorly differentiated HCCs (Table 2). However, there was no significant difference in gelatinase B expression among the histological grades. Transcripts for gelatinase B were also observed in a dysplastic nodule (1 of 2 cases) and in a replacing pattern of HCCs (Figure 1, D–F).

Strong expression of gelatinase B was observed in cancer cells at the invasion sites of both capsules (Figure 2, A and B) and portal veins. Of 13 cases with capsular invasion of HCCs, 12 expressed gelatinase B, whereas the expression was found in 10 of 14 cases without capsular invasion (Table 2; $P < 0.05$). Gelatinase B transcripts were also found in mesenchymal cells in fibrous capsules and around the necrosis of cancer nodules (Figure 2C).

Immunohistochemical study was also performed for identifying the cellular localization of protein of gelatinase B. Distribution of gelatinase B protein was mostly homogeneous and was similar to that of the transcripts (Figure 1, E and F). However, in some nodules, distribution of the protein was heterogeneous; stronger expression was found at the periphery or in some areas of nodules (Figure 2D).

In noncancerous liver, transcripts for gelatinase B were observed in sinusoidal cells of hepatic lobules and in mesenchymal cells of portal tracts (Figure 3A). There were some undefined cells that also expressed gelatinase B (Figure 3A). Weak gelatinase B expression was sometimes found in hepatocytes at the periphery of the hepatic lobules. Hybridization with sense probe as a negative control disclosed no significant signal (Figure 3B).

Gelatinolytic activities at both 92 and 65 kd were detected in cancer nodules as well as in noncancerous liver by a gelatin zymogram analysis (Figure 4).

Discussion

The cellular localization of gene transcripts and protein of gelatinase B in human HCCs was demonstrated in the present study. Transcripts for gelatinase B are frequently observed in cancer cells at an early stage of HCC and even in dysplastic nodules. These findings suggest that expression of gelatinase B is an important characteristic of malignant transformation of hepatocytes. The distribution of gelatinase B transcripts in cancer nodules is mostly homogeneous, but the signal intensity varies with the nodule. HCC is frequently surrounded by fibrous capsules, and capsular invasion of cancer cells is one of the most important prognostic factors of HCC.[22] Gelatinase B degrades denatured collagens and has been reported to digest some components of interstitial (type I and III) collagens,[9] the major content of hepatic collagens and also of the capsule of HCC. Therefore, gelatinase B may determine the invasive potential of HCCs.

The present study reveals that expression of gelatinase B in HCCs with capsular invasion was significantly more frequent than those without capsular invasion. Furthermore, strong expression of gelatinase B is observed in cancer cells at the invasion site of the fibrous capsule. These findings suggest that gelatinase B plays an important role in the invasion of HCC and the expression may relate to the prognosis of patients with HCC.

Immunohistochemical studies have demonstrated that in many cancer nodules the localization of gelatinase B protein is similar to that of the transcripts. However, in some cases the expression of the protein is heterogeneous, that is, stronger at the periphery and also in some parts of the nodules, whereas the transcripts are distributed homogeneously. Gelatinase B biosynthesis is increased in response to cytokines and growth factors, such as interleukin-1, tumor necrosis factor-α, epidermal growth factor, and platelet-derived growth factor. This increase has been reported to be due to the increased transcrip-

Figure 1. *Localization of transcripts and the protein for gelatinase B in HCCs and a dysplastic nodule.* **A:** *In HCC nodules, gene transcripts for gelatinase B are observed in cancer cells. Mesenchymal cells in and out of cancer nodules also express gelatinase B mRNA.* **B:** *In nodules of HCC, distribution of the transcripts is homogeneous in each cancer nodule, but signal intensity varies among different nodules.* **C:** *H&E staining of dysplastic nodule. Right half shows dysplasia, which existed in a nodule of well differentiated HCC, and left half is noncancerous liver.* **D:** *In a serial section of* **C**, *gelatinase B protein is demonstrated in a dysplastic nodule. The protein is also observed in white blood cells and some undefined cells at the periphery of a noncancerous hepatic lobule.* **E** *and* **F:** *In a replacing pattern of HCC, transcripts* (**E**) *and the protein* (**F**) *for gelatinase B are homogeneously observed in cancer cells. The distribution of the protein is similar to that of the transcripts.*

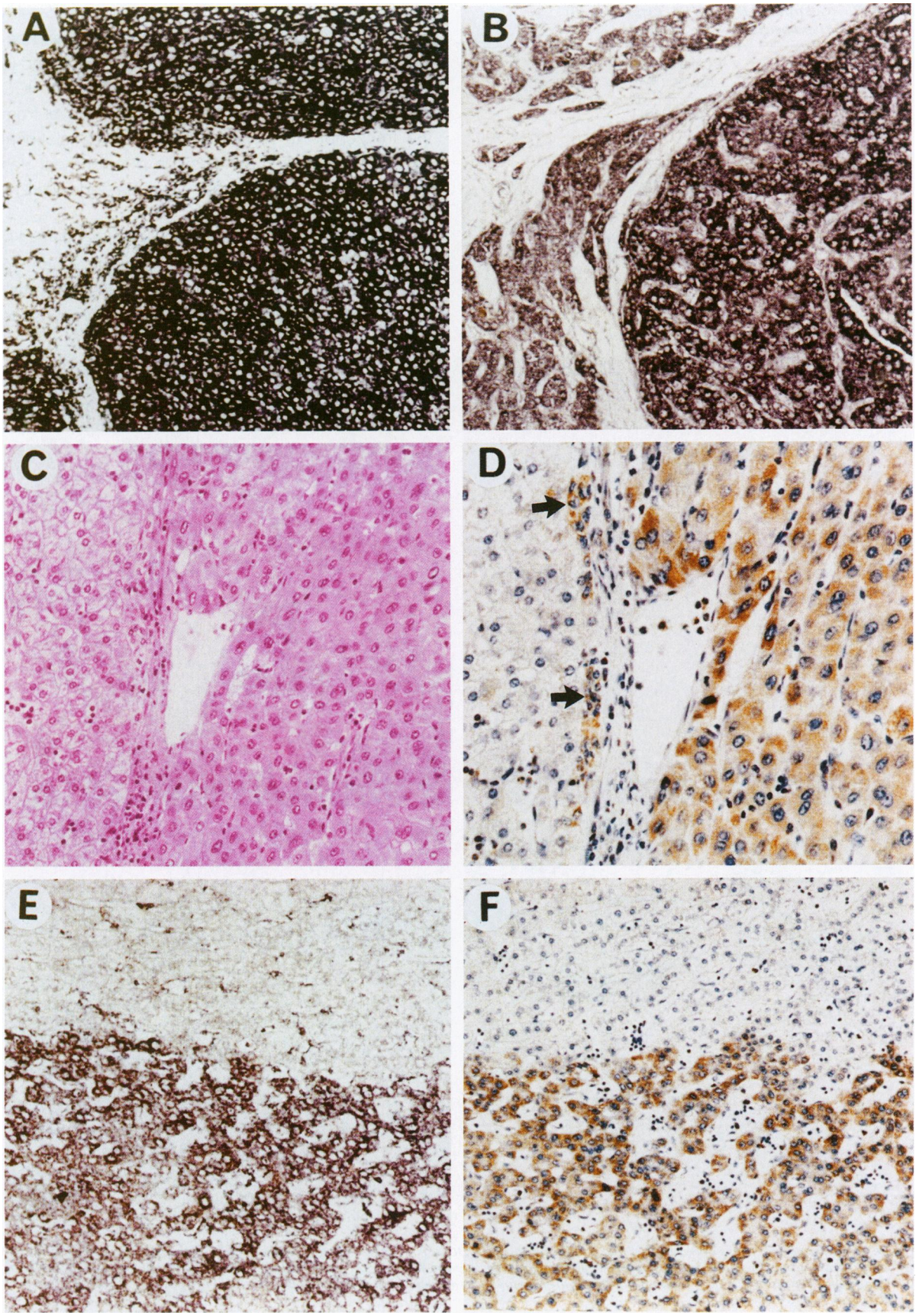
A
B
C
D
E
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Table 2. *Gelatinase B-9 mRNA Expression in Hepatocellular Carcinoma*

	Positive/total cases (%)	Significance	
Total	22/27 (81%)		
Differentiation grade of HCC			
Well	6/8 (75%)	NS	NS
Moderate	13/15 (87%)		
Poor	3/4 (75%)	NS	
Capsular invasion			
+	12/13 (92%)	$P < 0.05$	
–	10/14 (71%)		

NS, not significant.

tion by the action upon the promoter region of gelatinase B.[6,23,24] However, the discrepancy of expression between the transcripts and its protein found in the present study suggests that gelatinase B in HCC cells is also regulated at the translational level from the transcripts to the protein. Altered translational regulation of protein synthesis in development has been reported.[25] Similar regulation may exist in HCC cells.

The precise mechanisms and roles of gelatinase B in HCC invasion have to be elucidated in conjunction with tissue inhibitors of metalloproteinases, other matrix metalloproteinases, and some other proteinases

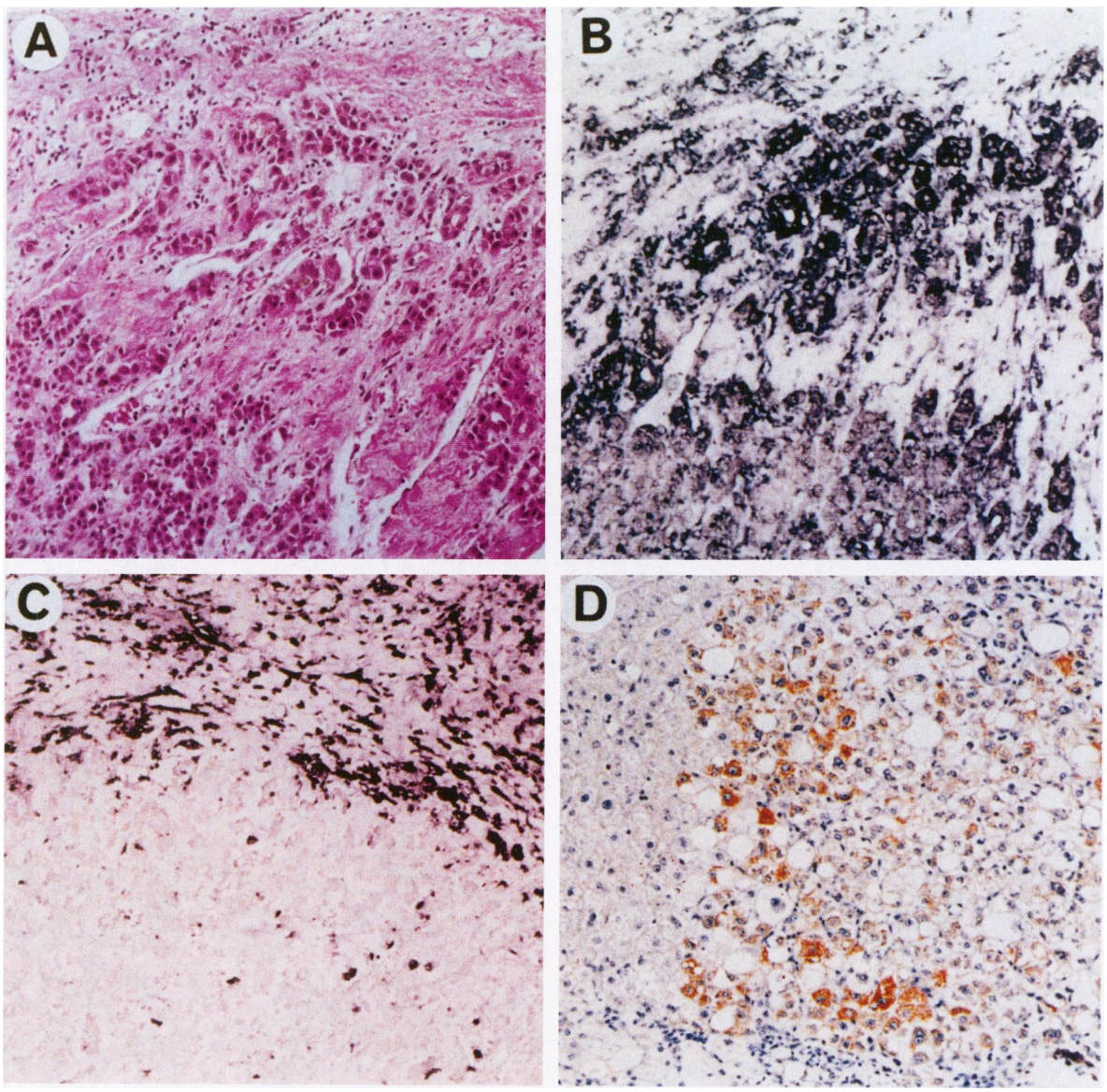

Figure 2. *Localization of transcripts and the protein for gelatinase B in HCCs.* **A:** *A cluster of HCC cells invaded into the capsule of an HCC nodule is shown. H&E staining.* **B:** *In a serial section of* **A**, *a strong signal of gelatinase B transcripts is demonstrated in cancer cells at the capsular invasion.* **C:** *Around the necrosis in HCC, gelatinase B transcripts are also found in mesenchymal cells.* **D:** *Strong expression of gelatinase B protein is observed at the periphery of a cancer nodule.*

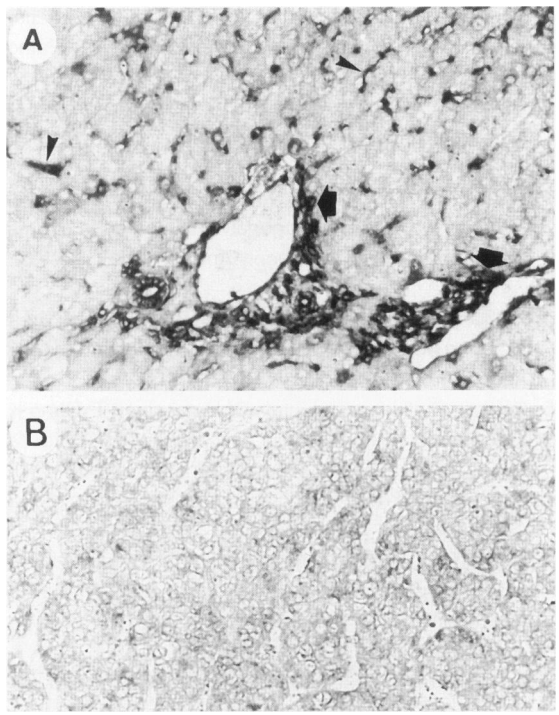

Figure 3. *Localization of transcripts for gelatinase B in noncancerous liver and hybridization with sense probe.* A: *Transcripts for gelatinase B are observed in sinusoidal cells in hepatic lobule and mesenchymal cells of portal tracts.* B: *The staining with the sense probe is not significantly different from nonspecific background level. Original magnification, ×100.*

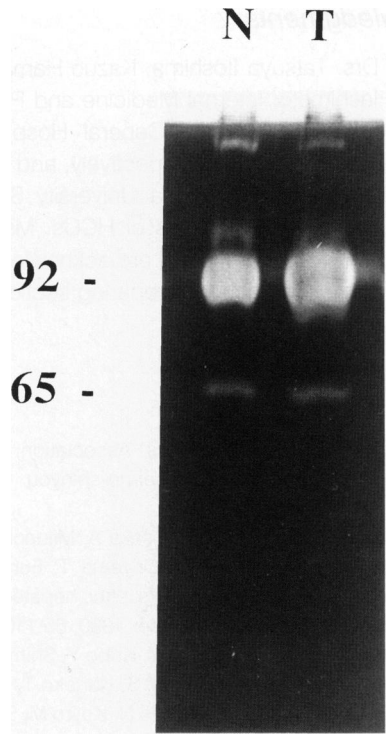

Figure 4. *Gelatin zymogram. Gelatinolytic activity was analyzed by a gelatin zymogram (see Materials and Methods). Ten micrograms (protein concentration) of the supernatant from liver homogenates was separated in a SDS gel containing 0.1% gelatin. Bands of gelatinolytic activity were visualized by negative staining of Amido Black. Gelatinolytic activities at both 92 kd and 65 kd in cancer nodules are detected as well as in noncancerous liver. N, noncancerous liver; T, tumor (HCC).*

such as plasminogen activator, but little is known.[26] Among these, the tissue inhibitors of metalloproteinases may be a major regulator of active gelatinase B. We have recently reported strong expression of tissue inhibitors of metalloproteinases-1 and -2 in HCCs.[27] However, the present gelatin zymogram reveals the activity of gelatinase B in HCCs, suggesting an important role in growth and invasion of cancer cells.

Increased transcripts for gelatinase B have been found in tumor inflammatory infiltrates of monkey HCCs and at the periphery of necrotic nodules of lung metastasis.[28] Gelatinase B may participate in extracellular matrix remodeling in cancer tissues. The present study also demonstrated the expression of gelatinase B in mesenchymal cells in fibrous capsule and at the periphery of necrotic cancer nodules. Gelatinase B may act on remodeling of extracellular matrix and also on the repair process of the necrotic tissue in cancer nodules.

Previous reports have shown that cultured Kupffer cells and lipocytes[29,30] expressed gelatinase B. The present study demonstrated that some other cell types in liver also express gelatinase B. In noncancerous liver, the protein and transcripts of gelatinase B are observed in at least three kinds of cells: sinusoidal lining cells in the hepatic lobules, mesenchymal cells in portal tracts, and some undefined cells at the edge of the hepatic lobules as well as in the portal tracts. These cells may play a role in degrading collagens around the hepatic lobules for maintaining the hepatic architecture. Most cells in the sinusoids expressing gelatinase B may be Kupffer cells and lipocytes. However, additional studies are needed to identify these cells.

As gelatinase B is highly expressed in HCCs and dysplasia, this enzyme can be a marker for early detection of HCCs and for differential diagnosis. Elevated levels of serum gelatinase B has been reported in patients with cancers in the gastrointestinal tract and breast.[31] We have previously reported elevated plasma levels of type IV collagenolytic activity in patients with portal invasion of HCC by using a substrate degradation assay.[32] However, whether the serum gelatinase B and type IV collagenolytic activity are derived from cancers is unknown. It is of importance to elucidate this issue as well as the correlation between the level of gelatinase B expression in HCC and the concentration in serum.

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