Expression of matrix metalloproteinases (MMPs) in cultured hepatocellular carcinoma (HCC) cells and surgically resected HCC tissues

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Abstract. Matrix metalloproteinases (MMPs) relate to the growth and infiltration of cancer cells, but the frequency and amount of their expression are not yet fully examined in hepatocellular carcinoma. Expression of MMPs (MMP-2, MMP-7, MMP-9, MT1-MMP, MT2-MMP, MT3-MMP) and tissue inhibitors of metalloproteinase (TIMP: TIMP-1, TIMP-2) was investigated on cultured hepatocellular carcinoma (HCC) cells and surgically resected HCC tissues. The cultured cells and tissues expressed MMPs and TIMPs at various degrees, and high expression was observed for MMP-2, MMP-9, MT1-MMP and TIMP-2. Expression of MMP-7, MT2-MMP and TIMP-1 was found at a low frequency and a low amount in both the cells and the tissues. MMP-2 was expressed in various cells: HCC cells, vascular wall and sinusoidal endothelial cells in the cancer area of surgically resected tissues; and hepatocytes, bile duct cells, vascular wall, macrophages and Kupffer cells in the non-cancerous area. MMPs and TIMPs were expressed at a relatively high frequency in hepatocytes of the cancerous area and surrounding non-cancerous area as well as in the other cells and tissues. MMPs and TIMPs may be involved in the progression of hepatocellular carcinoma including the infiltration of cancer cells.

Introduction

Tumor cells during their progression obtain high capability of growth, invasion and metastasis (1), and they change into highly malignant cells. Recent studies demonstrated that tumor cells require protease activities that resolve extracellular matrix in order to infiltrate into normal tissues, and this fact attracted attention to a family of endoproteinases, i.e. matrix metalloproteinases (MMPs) whose enzymatic activity is directed against components of the extracellular matrix. Invasive spread or infiltration of cancer cells needs resolution of extracellular matrix, and this is the result of the impaired balance between several MMPs and proteinase inhibitors called the tissue inhibitor of metalloproteinases (TIMP) (2). MMPs are produced not only by cancer cells but also stromal fibroblasts, infiltrating macrophages and granulocytes. MMP-2 and MMP-9 are known to be directly involved in degradation of extracellular matrix and of specific basement membrane molecules. In fact, various malignant tumors have abnormal MMP expression on their stroma. When tumor cells are unable to express secretory-type MMPs, they induce MMP production on the host cells. Tumor cells could specifically activate catalytic function of certain MMPs secreted by host cells by using proteases and other MMPs (3) and they utilize the activated MMPs for their invasion. MT-MMPs and MMP-3 on the other hand activate movement and angiogenesis of cancer cells, and this then induces effective invasion to the tissues. Therefore, clarification of the production and activation of MMPs is expected to provide a novel means that would prevent cancer growth and metastasis. We investigated the expression of MMPs and their inhibitors TIMPs in hepatocellular carcinoma (HCC) cell lines and surgically resected HCC tissues.

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Materials and methods

Cell lines and cell cultures. This study utilized 11 human HCC cell lines that were originally established in our laboratory, i.e. KIM-1 (4), KYN-1 (5), KYN-2 (6), KYN-3 (Murukami T, *et al*, Jpn J Cancer Res 292: abs. 1988), HAK-1A, HAK-1B (7), HAK-2 (8), HAK-3 (9), HAK-4, HAK-5 and HAK-6. These cell lines were previously confirmed to retain morphological and functional characteristics of the original HCC. KIM-1, KYN-1, HAK-2 and HAK-3 were established from surgically resected moderately differentiated HCC nodules; KYN-2 and HAK-6, from surgically resected moderately to poorly differentiated HCC nodules; and KYN-3, HAK-4 and

Table I. Results of flow cytometric analyses on the expressions of MMPs and TIMPs in HCC cell lines.

Cell	MMP-2	MMP-7	MMP-9	MT1-MMP	MT2-MMP	MT3-MMP	TIMP-1	TIMP-2
KIM-1	+++	±	+++	±	-	-	-	±
KYN-1	++	-	++	±	-	-	-	+
KYN-2	+++	-	+++	±	-	-	-	±
KYN-3	+++	-	+++	±	-	-	-	++
HAK-1A	+++	-	+++	+	-	-	-	+
HAK-1B	+++	-	+++	±	-	-	-	±
HAK-2	++	-	++	+++	-	-	-	+
HAK-3	+++	-	+++	+++	±	-	-	±
HAK-4	+++	±	+++	++	±	-	-	+
HAK-5	+++	±	+++	±	-	-	±	+
HAK-6	+++	-	+++	±	-	-	-	+

-, negative, positive cells accounted for <5% in total area; ±, weakly positive, 5-<25%; +, moderately positive, 25-<50%; ++, strong positive, 50-<75%; +++, very strong positive, ≥75%.

HAK-5, from peritoneal effusion of HCC patients with moderately to poorly differentiated HCC, poorly differentiated HCC, and sarcomatous HCC, respectively. HAK-1A and HAK-1B were 2 clonally related HCC cell lines established from a single HCC nodule showing a 3-layered structure with a different histological grade in each layer. HAK-1A is morphologically a well-differentiated HCC cell line, while HAK-1B is a poorly differentiated HCC cell line and biologically more malignant than HAK-1A, and is presumed to be derived from HAK-1A through its clonal dedifferentiation.

Tissue samples of HCC and non-HCC were obtained from 21 surgically treated HCC patients, and all specimens were obtained at Kurume University Hospital between 1996 and 2000. Informed consent was obtained from each patient who enrolled in the study. Tissue samples were used for immunohistochemistry, enzyme linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) analyses for MMPs and TIMPs. Each cell line was examined with flow cytometry and RT-PCR for MMPs and TIMPs.

Immunohistochemistry. Immunohistochemical staining of MMPs (MMP-2, MMP-7, MMP-9, MT1-MMP, MT2-MMP and MT3-MMP) and TIMPs (TIMP-1 and TIMP-2) were conducted by using catalyzed signal amplification system kits (Dako, CA) on paraffin sections of HCC tissues and their surrounding non-HCC tissues obtained from 18 cases (or 15 cases for MMP-7 examination). Primary antibodies were monoclonal antibody against each of the above-mentioned 6 human MMPs and 2 TIMPs (final dilution: 1/100-1/200, Daiichi Fine Chemical Co., Ltd., Tokyo, Japan). Peroxidase reaction was developed by using 3,3-diaminobenzidine tetrahydrochloride and the cells were counterstained with hematoxylin. The specimen was evaluated as having either: i) equivalent expression, i.e. HCC cells or non-neoplastic hepatocytes were stained as intensively as that of the positive internal control, ii) weak expression, i.e. the staining was less intensive, iii) high expression, i.e. they were more intensive or iv) negative expression, i.e. they were not stained at all.

ELISA. Portions of the surgically obtained HCC and non-HCC tissues were cut into pieces, and an appropriate amount was homogenized in 500 μl of ice-cold Ca^{++}-and Mg^{++}-free phosphate-buffered saline containing 100 μl/ml phenymethylsulfonyl fluoride by using a pellet pestle. The mixture was centrifuged for 10 min (12,000 rpm, 4°C), and the supernatant was stored at -20°C until use. The amount of tissue protein was determined by using the BCA protein assay reagent (Pierce Rockord, IL). The amount of active-MMP-2 and of pro-MMP-2 plus active-MMP-2 in supernatant was measured by using ELISA kits (Amersham International plc, Buckinghamshire, England). The amount of active-MMP and of pro-plus active-MMPs was assessed by making a correction of the measured level for the amount of protein.

Sixteen of the 18 cases whose tissues were examined in ELISA were also examined in the immunohistochemical staining, and they consisted of 3 cases of well + moderately differentiated HCC, 9 of moderately differentiated HCC and 4 of moderately + poorly differentiated HCC.

Flow cytometric analysis. Flow cytometric analyses for the MMPs and TIMPs were performed as described in our previous report (10). Antibodies used in the current study were monoclonal anti-MMP antibodies (final dilution, 1/50), anti-TIMP antibodies (final dilution, 1/50), and FITC conjugated goat anti-mouse IgG (Becton Dickinson Immuno-cytometry System USA, San Jose, CA), and analyses were done by using a FACScan (Becton Dickinson Immuno-cytometry System USA).

Analyses of MMP and TIMP mRNAs with reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA of cultured cells and tissues were extracted by using RNA-Bee™ (TEL-TEST, Inc., Friendswood, TX). RT-PCR for MMPs and TIMPs was performed as described in our previous report (10). PCR reaction was made with a primer specific to either MMP-2, MMP-7 (11), MMP-9 (12), MT1-MMP, MT2-MMP, MT3-MMP (13), TIMP-1, TIMP-2 (12)

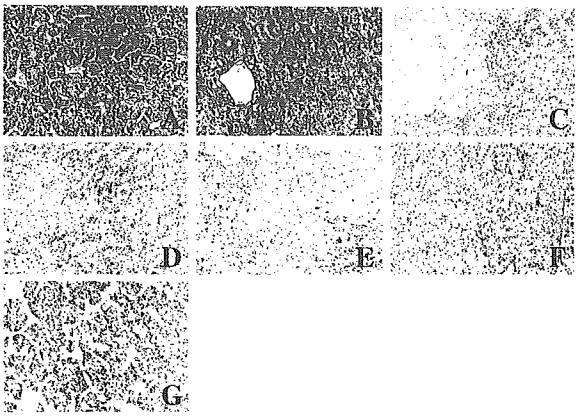

Figure 1. Immunohistochemical staining of MMPs and TIMPs. (A) MMP-2 was stained in the cytoplasms of neoplastic hepatocyte and sinusoidal endothelial cells in HCC. (B) MMT-2 was stained in the cytoplasm of non-neoplastic hepatocytes and in macrophages and Kupffer cells. (C) MMT-2 was stained predominantly on the cell surface of neoplastic hepatocytes at the boundary between HCC and non-HCC areas. (D) MMP-9 was stained in the cytoplasm of neoplastic hepatocytes in HCC. (E) MMP-9 was stained in the cytoplasms of non-neoplastic hepatocytes and sinusoidal lining cell in HCC. (F) MT3-MMP was stained in the cytoplasm of neoplastic hepatocytes in HCC. (G) TIMP-2 was stained in the cytoplasm of neoplastic hepatocytes in HCC. Counterstained with Mayer's hematoxylin. Original magnification, x100.

or ß-actin (14). PCR reaction was repeated 30-40 cycles by using iCycler (Bio-Rad Laboratories, CA), and one cycle consisted of denaturation, annealing and extension. PCR product (5 µl) was electrophorased with a 2% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) which contained 0.5% ethidium bromide, and specific DNA bands were examined under a UV transilluminator.

Results

Flow cytometric analyses. All cell lines expressed MMP-2 and MMP-9 at a high amount and frequency, i.e. ≥75% cells were positive. TIMP-2 and MT1-MMP were expressed but generally at a low amount and frequency, i.e. ≤50% cells were positive. Expression of MMP-7, TIMP-1, MT2-MMP and MT3-MMP as detected in 1-3 cell lines and the positive rate was also low (Table I).

Immunohistochemistry of surgically resected tissues. In the non-HCC tissues, MMPs and TIMPs were expressed on the hepatocytes, bile duct, vascular wall and metaplastic hepatocytes, showing ductular structures at the periphery of hepatic lobules; MMP-2, TIMP-2 and MMP-9 were also expressed on the Kupffer cells and macrophages; and MMP-2 was also expressed on the stromal connective tissues in the cases with abundant fibrosis. In the HCC tissues, MMP-2 was expressed on the HCC cells, sinusoidal endothelial cells and other lining cells, and fibrous capsule; and MMP-2, MMP-7, MMP-9 and MT3-MMP expression in some cases was more apparent on the HCC cells at the boundary between HCC and non-HCC areas. In the case of MMP-2, the expression was sometimes clearer on the cell surface than in the cytoplasm (Fig. 1).

In the 18 cases of HCC tissues, the highest frequency was noted for TIMP-2 (18/18, 100%), and the lowest frequency was noted for TIMP-1 (8/18, 44%). In the non-HCC tissues, the highest frequency was obtained also for TIMP-2 (17/18, 94%), and the lowest was also TIMP-1 (8/18, 44%). TIMP-2 and MMP-2 were expressed at a high frequency and a high

Table II. Immunohistochemical staining for MMTs and TIMPs in cancerous and non-cancerous tissues according to histological grade.

	Well + Mod (n=4)		Mod (n=9)		Mod + Poor (n=5)		Total (n=18)	
	C (%)	N (%)	C (%)	N (%)	C (%)	N (%)	C (%)	N (%)
MMP-2	4 (100)	4 (100)	8 (89)	8 (89)	5 (100)	4 (80)	17 (94)	16 (89)
MMP-7*	3 (75)	3 (75)	4 (44)	6 (67)	1 (20)	2 (40)	8 (53)	11 (73)
MMP-9	3 (75)	2 (50)	7 (78)	6 (67)	3 (60)	4 (80)	13 (72)	12 (67)
MT1-MMP	3 (75)	3 (75)	6 (67)	6 (67)	3 (60)	5 (100)	12 (67)	14 (78)
MT 2-MMP	2 (50)	2 (50)	5 (56)	5 (56)	2 (40)	3 (60)	9 (50)	10 (56)
MT 3-MMP	2 (50)	2 (50)	6 (67)	6 (67)	4 (80)	5 (100)	12 (67)	13 (72)
TIMP-1	2 (50)	2 (50)	5 (56)	4 (44)	1 (20)	2 (40)	8 (44)	8 (44)
TIMP-2	4 (100)	4 (100)	9 (100)	8 (89)	5 (100)	5 (100)	18 (100)	17 (94)

Well: well-differentiated HCC. Mod: moderately-differentiated HCC. Poor: poorly-differentiated HCC. n: number of HCC tissues examined. C: cancerous tissues. N: non-cancerous tissues. *Total number was 15 (4 Well+Mod, 7 Mod and 4 Mod+Poor).

Table III. Active or total MMP-2 protein levels in surgically resected HCC and its continuous areas: Results of ELISA.

Histologic grade	MMP-2 protein	HCC tissue	Non-HCC tissue
Well + Mod (n=3)	Total (pro + active)	0.92 ± 0.15	0.48 ± 0.05
	Active	0.00	0.00
Mod (n=10)	Total (pro + active)	0.69 ± 0.52	0.87 ± 0.55
	Active	0.01 ± 0.01	0.03 ± 0.03
Mod + Poor (n=4)	Total (pro + active)	0.66 ± 0.63	1.13 ± 0.43
	Active	0.02 ± 0.02	0.06 ± 0.60
Poor (n=1)	Total (pro + active)	4.09	0.47
	Active	0.00	0.00
Total (n=18)	Total (pro + active)	0.91 ± 0.92	0.84 ± 0.50
	Active	0.01 ± 0.02	0.03 ± 0.04

Data are expressed as ng/20 μg protein. Well: well-differentiated HCC. Mod: moderately-differentiated HCC. Poor: poorly-differentiated HCC.

amount, and there were no remarkable differences between HCC and non-HCC tissues. On the other hand, the expression of TIMP-1 and MT2-MMP was low in frequency and amount. In the comparison of MMP and TIMP expression between neoplastic and non-neoplastic hepatocytes, frequencies of the expression were almost the same except MMP-2 that expression was higher in the neoplastic hepatocytes (Table II).

ELISA: Measurement of active and total (pro-active) MMP-2 proteins. The mean amount of total MMP-2 in the 18 cases was 0.91 ng/20 μg protein in the HCC area and 0.84 ng/20 μg protein in the non-HCC area. Amount of total MMP-2 protein was higher in the non-HCC area than in HCC area of the following 9 cases (50%), i.e. 6/10 (60%) moderately differentiated HCC cases and 3/4 (75%) moderately + poorly differentiated HCC cases. The remaining 9 cases (50%) had higher total MMP-2 in the non-HCC area than in HCC area, and the highest amount of total MMP-2 was found in the HCC area (4.09 ng/20 μg protein) of one poorly differentiated HCC (Table III). Active-MMP-2 was detected in the HCC and/or non-HCC areas of 8/18 cases. In the 9 cases whose total MMP-2 was higher in the non-HCC area, mean active-MMP-2 in the HCC area was 0.02 ng/20 μg protein and that in the non-HCC area was higher, i.e. 0.05 ng/20 μg protein.

RT-PCR. All cell lines clearly expressed MMP-2, MMP-9 and MT1-MMP mRNAs, and mRNAs of the other MMPs and TIMPs at various degrees. MMP-7 and MT3-MMP mRNAs were detected at a low frequency. All cases expressed MMP-2, MMP-7, MMP-9 and MT1-MMP mRNAs in both HCC and non-HCC tissues. Frequencies of MT2-MMP and TIMP-1 mRNA expression were low (Fig. 2).

Discussion

Our *in vitro* study showed that HCC cell itself expresses MMPs and TIMPs at various degrees. The expression of MMP-2 and MMP-9 was high in terms of frequency and

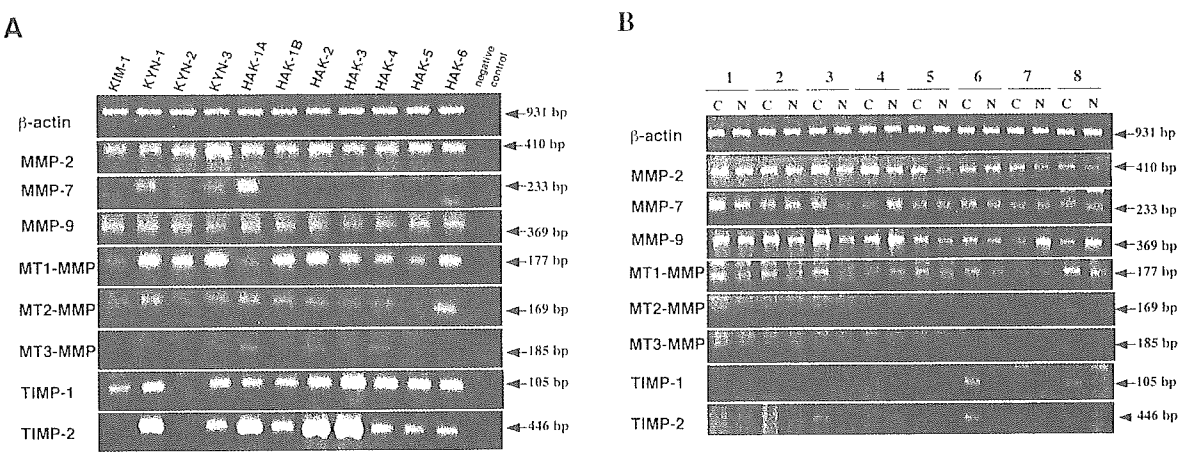

Figure 2. Expression of mRNAs of the MMPs and TIMPs in the HCC cell lines (A) and HCC tissues (B) as measured with RT-PCR technique. The PCR products were electrophoresed in a 2% NuSieve agarose gel and stained with ethidium bromide. In (B), case number is shown on the top. C: cancerous tissue. N: non-cancerous tissue. PCR reaction using ß-actin-specific primers showed that equal amounts of cDNA had been used and similarly amplified.

amount, and this was followed by MT1-MMP and TIMP-2. On surgically resected HCC tissues, the expression of MMP-2, MMP-9, MT1-MMP and TIMP-2 was high, and the *in vivo* and *in vitro* results corresponded well.

MMP-2 produces a complex with TIMP-2 as well as with MT1-MMP, and activation of pro-MMP-2 is usually inhibited by TIMP-2. MT1-MMP activates MMP-2 (15,16) and then the active-MMP-2 activates pro-MMP-9 (17). MMP-9 is also partly activated with MMP-7 (18). MMP-2 and MT1-MMP degrade the extracellular matrix and relate to invasion and metastasis of the cancer cells (19). MMP-2 in particular is the key enzyme that takes an important role in the invasion to basement membrane. It is shown that cancerous tissues with a high expression level of active-MMP have a high risk of metastasis. Therefore, the activation rate of pro-MMP-2 is used as an indicator of cancer metastasis (20). In regard to HCC, there are reports showing the involvement of MMP-2 and MMP-9 to capsular invasion (1,2), involvement of MMP-9 to portal vein invasion (21), high expression of MT1-MMP in poorly differentiated HCCs, and poor prognosis of the cases with MT1-MMP expression (17). In our immunohistochemistry, the 5 cases that contained poorly differentiated HCC (i.e. 4 moderately + poorly differentiated HCCs and 1 poorly differentiated HCC) expressed MT1-MMP in either or both of the cancerous and non-cancerous areas. The 8 cases in which ELISA showed the expression of active-MMP-2 did not contain well-differentiated HCC. These findings indicate that, in HCC, poorly differentiated HCC expresses TIMP-2 but this expression interacts with MT1-MMP and this makes an activation system for MMP-2.

Cytokines such as TNF-α are reported to activate MMP-2 and MMP-9 (22,23). TNF-α is also reported to enhance MT1-MMP expression (24). We therefore examined the effects of various cytokines on MMP-2 expression by using the HCC cell lines, but there were no noteworthy findings (data not shown).

In normal conditions, pro-MMP-2 and TIMP-2, and pro-MMP-9 and TIMP-1 produce a complex. MMP activity is inhibited by TIMP in 1:1 mol basis, and the activation into active-MMP-2 and active-MMP-9 is limited. In our surgically resected tissues and cultured HCC cell lines, the expressions of MMP-2 and TIMP-2 were equally observed, while expression of MMP-9 was higher than TIMP-1, causing an imbalance between MMP-9 and TIMP-1. Lichtinghagen *et al* (12) reported that TIMP-1 expression was higher than TIMP-2 in patients with hepatitis or liver cirrhosis. Hayashi *et al* showed MMP-9 expression elevated with aggravation of inflammation from hepatitis, liver cirrhosis to liver cancer (21). Sakamoto *et al* demonstrated the involvement of MMP-9 to HCC since its very early stage (25). MMP-9 is also known to be up-regulated with angiopoietin-2 and relate to angiogenesis (26,27). Mechanism of MMPs action on carcinogenesis and tumor growth is thought to be different according to the origin of the tumor, e.g. TIMP-1 was expressed but TIMP-2 was rarely expressed in thyroid carcinoma (28) and a hamster model of pancreatic duct carcinoma expressed only a low amount of TIMP-1 (29). In our findings, MMP-9 expression was obvious but TIMP-1 expression was low. Therefore, the balance between MMP-9 and TIMP-1 would be distorted in the carcinogenesis process of the liver, and MMP-9 could affect angiogenesis at the early stage of HCC and then the growth and infiltration of HCC cells.

The HCC cell lines expressed MT1-MMP but rarely expressed MT2-MMP and MT3-MMP. On the HCC tissues, MT1-MMP was expressed on the bile duct and metaplastic hepatocytes that showed ductular structures at the periphery of hepatic lobules, stromal cells and HCC cells. However, MT2-MMP expression on the tissue was low in its frequency and amount. MT3-MMP expression on the tissues was relatively high, and found on HCC cells, metaplastic hepatocytes that showed ductular structures, bile duct and vascular wall. The findings on MT1-MMP and MT2-MMP in the tissues agreed with our *in vitro* findings, but MT3-MMP results did not.

Inducers and suppressors of MT3-MMP are not yet well-known, but Lafleur *et al* (30) reported that MT-MMP expression is affected by the expression of MMP inhibitors, growth factors that relate to angiogenesis, and cytokines. In our findings, the results on MT3-MMP expression did not agree, between cultured HCC cells and surgically obtained HCC specimens, but this disagreement suggested that MT3-MMP expression in the tissue was the result of interaction between various cells including HCC cells on paracrine and autocrine bases. MT1-MMP and MT3-MMP are known to enhance fibrin-invasive activity, and MT1-MMP, MT2-MMP and MT3-MMP are related to endothelial tubulogenesis (30). In our tissue examination, endothelial cells were positive to these 3 MMPs, suggesting that these MMPs most probably take part in tubulogenesis as well as directly participate in cell proliferation and invasion (31,32).

Yamamoto *et al* (33) reported that MMP-7 relates to cancer progression, its expression in gastric cancer is higher in well-differentiated adenocarcinoma, and it plays an important role in the infiltration of cancer cells through its resolving activity on extracellular matrix. In our cases, MMP-7 expression was lower in frequency and amount in the tissues containing poorly differentiated HCC than those containing well-differentiated HCC. The expression was also low in the HCC cell lines. This suggests that MMP-7 relates to cancer progression in the early stage. MMP-7 is also reported to take part in the invasion of gastric cancer and the metastasis of lymph nodes, and it could be used as a predictive factor of prognosis (34). Cholangiocellular carcinoma expressing MMP-7 was reported to have high malignancy and short survival period (35-37). Expression of MMP-7 needs to be investigated further.

MMPs and TIMPs were expressed in cancerous and non-cancerous areas of HCC in various degrees. MMPs were thought to be activated as a result of quantitative and qualitative imbalance of these expressions, and then to take part in proliferation, invasion and metastasis of HCC. Our findings indicate that blockage of the mechanism of MMP action would be an efficacious means to prevent or suppress development and proliferation of HCC.

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Expression and activation of apoptosis-related molecules involved in interferon-α-mediated apoptosis in human liver cancer cells

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Abstract. Interferon (IFN)-α directly inhibits proliferation of liver cancer cells by inducing apoptosis, but the molecular mechanisms by which IFN-α induces apoptosis in these cells are not fully understood. We examined the effect of broad spectrum caspase inhibitor, Z-VAD-fmk, and the caspase activation in IFN-α-mediated apoptosis by using 4 liver cancer cell lines that were sensitive or resistant to IFN-α-mediated apoptosis. Involvement of apoptosis-related mitochondrial proteins and Bcl-2 family proteins in IFN-α-mediated apoptosis was further examined in 1 sensitive cell line (KIM-1). The Z-VAD-fmk completely or moderately inhibited IFN-α-mediated apoptosis in the sensitive cells. IFN-α induced time-dependent activation of caspase-3 in the sensitive cells, while the resistant cells showed mild or no activation. Activation of caspase-9, caspase-8, and caspase-7, and the cleavage of poly(ADP-ribose)polymerase were identified in either or both of the sensitive cell lines, but not in the resistant cells. In KIM-1 cells, the release of cytochrome c and Smac/DIABLO from mitochondria to cytosole was confirmed. Meanwhile, Bcl-x_L was upregulated, and Bid activation or translocation, or conformational changes of Bax were not identified. In conclusion, our results suggest IFN-α-mediated apoptosis in liver cancer cells involves the mitochondrial apoptotic pathway and is induced by activating various caspases.

Introduction

Interferon (IFN)-α has various biological actions such as antivirus, antiproliferative, immunomodulatory, anti-telomerase and anti-angiogenesis effects (1,2). Their antivirus effects are utilized in a treatment for chronic hepatitis C; and the antiproliferative effects, for malignant diseases such as leukemia and renal cancer (3). *In vitro* experiments of the direct antiproliferative effects of IFN-α demonstrated the induction of apoptosis and cell-cycle arrest at G_1/S, S or G_2/M phases in many cell lines (4-10).

Induction of apoptosis mechanism would be initiated through 2 major signaling pathways, i.e., death receptor pathway (11) and a mitochondrial pathway (12-16). Recent studies (17-20) suggest the involvement of the mitochondrial pathway in IFN-α or -ß-mediated apoptosis, however, not all the findings are consistent. The mitochondrial pathway is initiated at the mitochondria by the release of apoptogenic factors, such as cytochrome c, and they trigger activation of effector caspases such as caspase-3 through the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, and eventual apoptosis with the cleavage of diverse cellular substrates, such as poly(ADP-ribose)polymerase (PARP) (13-15).

We previously reported that human natural IFN-α induced apoptosis *in vitro* at a relatively high frequency (77%, 10 of the 13 cell lines), and this apoptosis was not closely related to: i) the expression of Bcl-2 family members such as Bax, Bak, Bcl-2 and Bcl-x_L, ii) condition of the gene p53, and iii) the Fas/Fas ligand (10). More recently, we reported that IFN-α-mediated suppression of hepatocellular carcinoma (HCC) cell proliferation *in vivo* is related to the induction of apoptosis and the suppression of angiogenesis (21). In the present study, we investigated the involvement of apoptosis-related molecules, including mitochondrial proteins and caspases, by using liver cancer cell lines that were sensitive or resistant to IFN-α-mediated apoptosis.

Materials and methods

Cell lines and cell culture. This study utilized 3 HCC cell lines [KIM-1 (22), KYN-3 (Murakami *et al*, Jpn J Cancer Res Proceedings of the Japanese Cancer Association: abs. 292, 1988), HAK-1B (23)] and 1 human combined hepatocellular and cholangiocarcinoma cell line [KMCH-2 (24)]. In our previous study (10), we found that KIM-1 and HAK-1B were sensitive to IFN-α-mediated apoptosis, while the other 2 cell lines (KYN-3 and KMCH-2) were resistant to this apoptosis.

Each cell line was grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Co., Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 μg/ml

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streptomycin (Gibco BRL/Life Technologies, Inc., Gaithersburg, MD) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37°C.

Cytokines, antibodies, and reagents. Natural human IFN-α (OIF) was kindly provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Monoclonal anti-human/mouse cytochrome c antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Products from Cell Signaling Technology, Inc. (Beverly, MA) were: a biotinylated protein marker detection pack, Chaps Cell Extract buffer, Cell Lysis buffer, rabbit polyclonal antibodies to cleaved caspase-3, caspase-3, cleaved caspase-7, caspase-7, cleaved caspase-9, caspase-9, cleaved poly(ADP-ribose)polymerase (PARP), PARP, BID and Smac/DIABLO, and monoclonal antibody to caspase-8. Anti-human cytochrome oxidase subunit II mouse monoclonal 12C4-F12 antibody was purchased from Molecular Probes, Inc. (Eugene, OR); rabbit polyclonal antibodies to human Bax, N-termina, from Upstate Biotechnology (Lake Placid, NY); rabbit polyclonal anti-human Bax antibody (clone 4F11), from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan); rabbit polyclonal anti-human Bcl-x_L antibody, from Transduction Laboratories (Lexington, KY); monoclonal anti-ß-actin antibody, from Sigma Chemical Co. (Saint Louis, MO); Jurkat cell extract, non-induced and induced immunoblotting standards, from BioMol Research Laboratories Inc. (Plymouth Meeting, PA); and irreversible, cell permeable, broad spectrum caspase inhibitor, Z-Val-Ala-Asp (OMe)-Fluoromethylketone (Z-VAD-fmk), from Kamiya Biomedical Co. (Seattle, WA). Antibodies were used at the concentrations recommended by the manufacturers. The amount of protein in the samples was measured by using the BCA protein assay reagent (Pierce, Rockford, IL).

Effects of caspase inhibitor on IFN-α-mediated apoptosis. KIM-1 or HAK-1B cells ($2.0x10^5$ cells/well) were treated or untreated with 1,000 IU/ml IFN-α for 72 h and processed for assessment of apoptosis. Broad spectrum caspase inhibitor Z-VAD-fmk was used at a final concentration of 8, 20, or 50 μM and it was added to the culture medium 1.5 h prior to IFN-α treatment. For the control, vehicle (acetone) alone was added to the culture. For KIM-1 cells, an additional experiment was conducted, in which cultured cells were pretreated with Z-VAD-fmk at a final concentration of 40 or 100 μM, 1.5 h prior to the addition of IFN-α, cultured with IFN-α for 24 h, re-treated with the same amount of a fresh inhibitor, cultured for 24 h, and processed for assessment of apoptosis. Assessment of apoptosis was conducted by observation under a phase-contrast microscope (Nikon, Tokyo, Japan), and by terminal deoxynucleotidyl transferase (tdt)-mediated dUTP nick end-labeling (TUNEL) method. MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories Co., Ltd.) was used according to the manufacturer's recommendation. The stained cells were analyzed by using a FACScan (Becton Dickinson Immunocytometry Systems USA, San Jose, CA).

Assessment of caspase-3 and caspase-9 activities by fluorometric protease assay. Quantitative analysis on the activation of caspase-3 and -9 was conducted by using Fluorometric Protease Assay Kits (Medical & Biological Laboratories Co., Ltd.) according to the manufacturer's protocol. Briefly, the cellular protein (100 μg) obtained from the cells cultured with or without 1,000 IU/ml IFN-α for several intervals was incubated with appropriate caspase substrate in the reaction buffer at 37°C for 2 h. DEVD-AFC and LEHD-AFC substrates were used for the activity measurement of caspase-3 and -9, respectively. Caspase activities were estimated by measuring a yellow-green fluorescence with a Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) by setting the excitation filter at 400 nm and the emission filter at 505 nm.

Sodium dodecyl sulfate-polyacrylamide slab gel (SDS-PAGE) electrophoresis and Western blotting. We investigated the activation of caspase-3, -7, -8 and -9, and cleavage of PARP in the 4 cell lines cultured with or without 1,000 IU/ml IFN-α for 72 h. Cells were solubilized in Chaps Cell Extract buffer or Cell Lysis buffer for the detection of caspase-3, -7 and -9, and PARP according to the manufacturer's protocol. For caspase-8 detection, cultured cells were lysed by adding Laemmli's sample buffer. We also investigated the expression of cytochrome c, cytochrome oxidase subunit II, Bax, BID, Bcl-x_L, and Smac/DIABLO in mitochondrial and cytosolic fractions of KIM-1 cells cultured with or without 1,000 IU/ml IFN-α for 24, 48, or 72 h. The mitochondrial and cytosolic fractions were isolated from KIM-1 cells using the method described by Wang *et al* (25). The cellular proteins were subjected to electrophoresis in the 12.5% SDS-PAGE and transferred to polyvinylidine difluoride transfer membrane (Immobilon-P, Millipore Corporation, Bedford, MA) using the Trans-blot SD semi-dry transfer cell (Bio-Rad, Richmond, CA). Immunoblotting was performed using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK) as previously described (10). The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. The protein levels were normalized against ß-actin levels used as an internal control.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction and RT-PCR reaction were performed by using a technique described previously (10) with slight modification. After RT reaction, phenol (pH 8.0) extraction was performed twice, and purified cDNA was used as the template of DNA synthesis. PCR reaction of Apaf-1 and caspase-2, -3, -4, -5, -6, -7, -8, -9 and -10 was performed with Human Apoptosis Genes Set-5 and Set-6 MPCR Amplification Kits (Maxim Biotech. Inc., San Francisco, CA) according to the manufacturer's protocol. PCR reaction was made through 2 cycles of 94°C (1 min) and 62°C (4 min), followed by 30 cycles of 94°C (1 min) and 62°C (2.5 min), and 1 cycle of 70°C (3 min), by using a Thermocycler (Perkin-Elmer Cetus Corp., Norwalk, CT). PCR product (5 μl) was electrophoresed with a 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). The gel was stained with 0.1% ethidium bromide, and then specific DNA bands were examined under an ultraviolet transilluminator.

Results

Effects of caspase inhibitor on IFN-α-mediated apoptosis. Addition of 1,000 IU/ml of IFN-α to the cultures of KIM-1

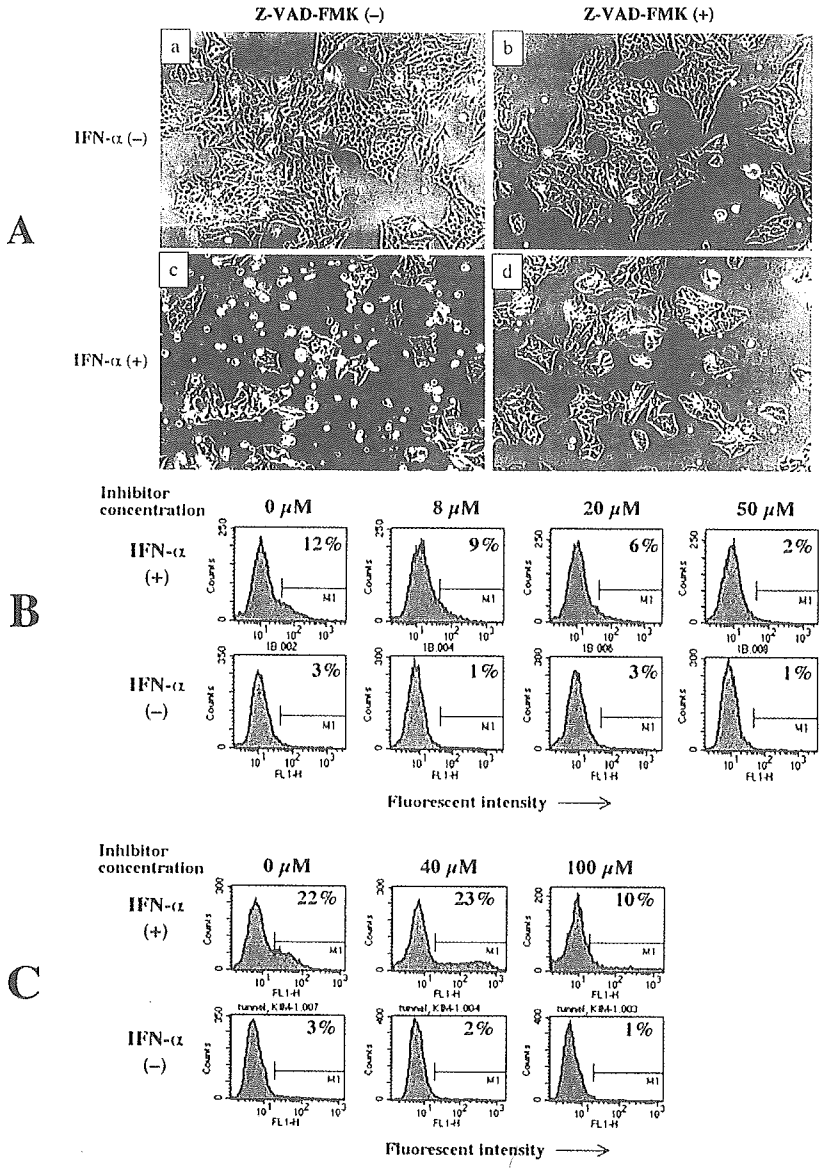

Figure 1. Effects of the broad spectrum caspase inhibitor Z-VAD-fmk on IFN-α-mediated apoptosis in HAK-1B cells (A and B) or KIM-1 cells (C) were assessed under a phase-contrast microscope (A) or by TUNEL method (B and C). In all experiments, Z-VAD-fmk was added to the medium 1.5 h before the addition of IFN-α. (A), HAK-1B cells that were pretreated with (b and d) or without (a and c) 50 μM Z-VAD-fmk were cultured with (c and d) or without (a and b) 1,000 IU/ml IFN-α for 72 h. (B), HAK-1B cells that were pretreated with 0, 8, 20 or 50 μM Z-VAD-fmk were cultured with or without 1,000 IU/ml IFN-α for 72 h, and TUNEL-positive cell rates were assessed by flow cytometry. The value shown with each histogram represents the percentage of TUNEL-positive apoptotic cells. (C), KIM-1 cells that were pretreated with 0, 40 or 100 μM Z-VAD-fmk were cultured with IFN-α for 24 h, re-treated with the same amount of a fresh inhibitor, cultured for another 24 h, and processed for assessment of apoptosis by TUNEL method. The value shown with each histogram represents the percentage of TUNEL-positive apoptotic cells.

and HAK-1B cells resulted in the induction of apoptosis from 48 h later, and the number of apoptotic cells increased over time. These findings agree with our previous results (10). In HAK-1B cells, the broad spectrum caspase inhibitor Z-VAD-fmk showed dose-dependent decrease of IFN-α-mediated apoptotic cells until 72 h after the IFN-α addition under a phase-contrast microscope (Fig. 1A). In addition, TUNEL-positive apoptotic cells acounted for 12% of the cells in the cultures without addition of the inhibitor, and the ratio decreased along with the dose-increase of the inhibitor, e.g. to 2% with 50 μM (Fig. 1B). The same experiment was performed for KIM-1 cells, but suppression of apoptosis was not observed under both assays (data not shown). We then used 100 μM of the inhibitor and the cells were re-challenged with fresh inhibitor after 24 h of the culture. Under phase-contrast microscope (data not shown), suppression of apoptosis was observed, and the TUNEL examination also showed that the rate of positive cells decreased from 22 to 10% (Fig. 1C).

Activation of caspases by IFN-α. In the fluorometric protease assay, activation of caspase-3 and -9 did not occur or occurred at a low level 24 h after the addition of IFN-α in the 2 cell lines

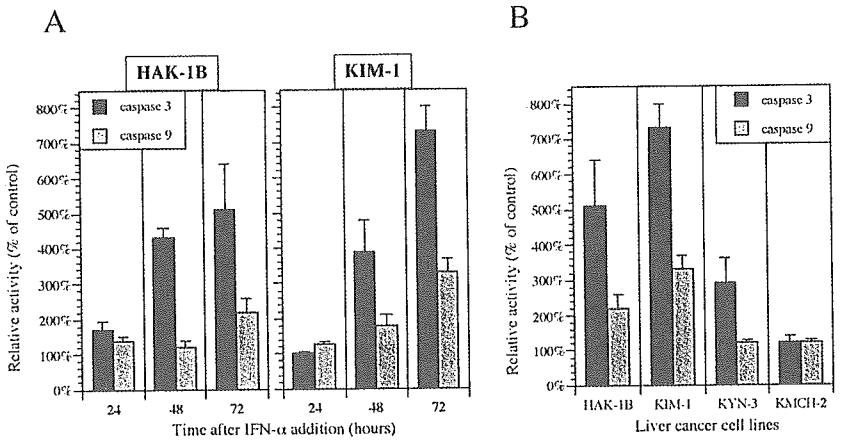

Figure 2. Effects of 1,000 IU/ml IFN-α on activation of caspase-3 and -9, analyzed by fluorometric protease assay. (A), Time-course change of relative activity (in comparison to the activity in the no-treatment group) of caspase-3 and -9 in the 2 IFN-α-mediated apoptosis-sensitive cell lines cultured with 1,000 IU/ml IFN-α. (B), Relative activity of caspase-3 and -9, 72 h after the addition of 1,000 IU/ml IFN-α to the cultures of the 2 sensitive cell lines (KIM-1 and HAK-1B) and 2 resistant cell lines (KYN-3 and KMCH-2). The experiment was repeated at least twice to confirm the reproducibility of the test results.

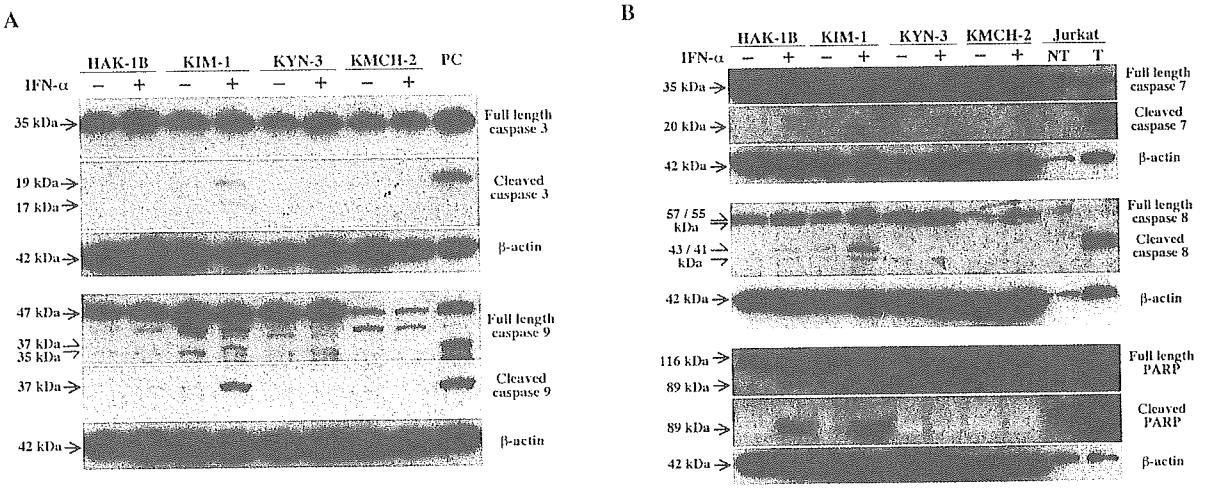

Figure 3. Effects of IFN-α on full-length expression and cleavage of caspase-3, -9 (A), -7 and -8, and PARP (B) in the 4 liver cancer cell lines, analyzed by Western blotting. Two IFN-α-mediated apoptosis-sensitive cell lines (KIM-1 and HAK-1B) and 2 resistant cell lines (KYN-3 and KMCH-2) were incubated with medium alone (-) or 1,000 IU/ml IFN-α for 72 h, and then used in analysis. Seventy-five μg proteins from each cell line were used for the detection of caspases-3, -7, and -9, and PARP. For caspase-8 detection, samples were prepared from $1.0x10^5$ KYN-3 cells, $1.5x10^5$ KIM-1 and HAK-1B cells, and $2.0x10^5$ KMCH-2 cells. In the analysis of caspase-7, caspase-8 and PARP, and for positive control of caspase and PARP cleavage, protein extracts from Jurkat cells untreated with the chemotherapeutic agent etoposide were used. The ß-actin level was used as the control for equal loading.

(HAK-1B and KIM-1) that are sensitive to IFN-α-mediated apoptosis. However, the activity levels increased with the contact hours, and the levels reached the highest at 72 h after IFN-α addition (Fig. 2A), when the activities were also measured in the other 2 cell lines (KYN-3 and KMCH-2) that are resistant to IFN-α-mediated apoptosis. As a result, the activity levels were 5.1 times higher in HAK-1B, 7.3 times in KIM-1, 2.9 times in KYN-3, and 1.2 times in KMCH-2, for caspase-3, and 2.2, 3.3, 1.2, and 1.2 times higher, respectively, for caspase-9 (Fig. 2B).

In the Western blot analysis, KIM-1 (sensitive cells) cultured with IFN-α had weak bands of cleaved caspase-3 and -7, and clear bands of cleaved caspase-8 and -9, while HAK-1B cells cultured with IFN-α had weak bands of cleaved caspase-3, -8 and -9 (Fig. 3). Both IFN-α-treated KIM-1 and HAK-1B had cleaved product of PARP (Fig. 3B). In the resistant cell lines (KYN-3 and KMCH-2), these activation bands were not found.

Activation of mitochondrial proteins and Bcl-2 family proteins by IFN-α. The expression of 2 mitochondrial proteins, i.e. cytochrome c and Smac/DIABLO, at 24, 48 and 72 h with or without IFN-α treatment was examined in IFN-α-mediated apoptosis-sensitive KIM-1 cells. As a result, the cytochrome c and Smac/DIABLO levels in the mitochondrial fraction decreased at 72 h after treatment, and cytochrome c level in

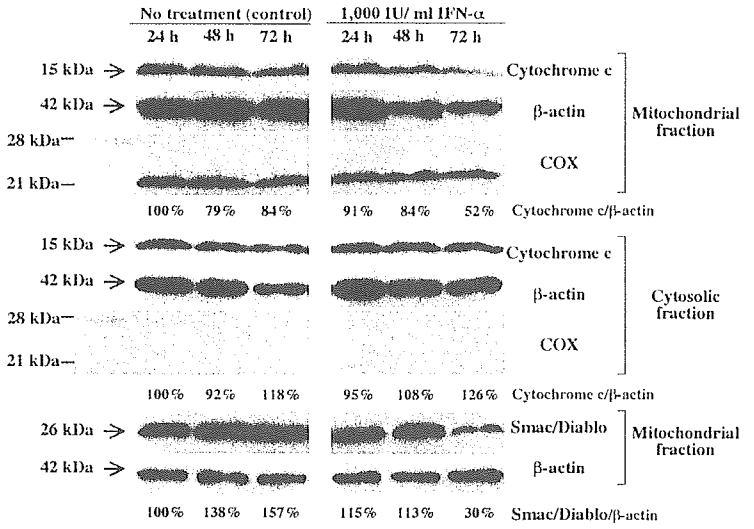

Figure 4. Effects of 1,000 IU/ml IFN-α on the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol in KIM-1 cells, analyzed by Western blotting. Equal amounts (50 μg) of protein were subjected to electrophoresis. Cytochrome oxidase subunit II (COX) was expressed in mitochondrial fraction, but not in cytosolic fraction. This shows that proteins in the cytosolic fraction were not intermixed with those in the mitochondrial fraction. The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. Cytochrome c and Smac/DIABLO levels were normalized against ß-actin levels that were used as an internal control. The normalized levels of cytochrome c and Smac/DIABLO were comparatively analyzed.

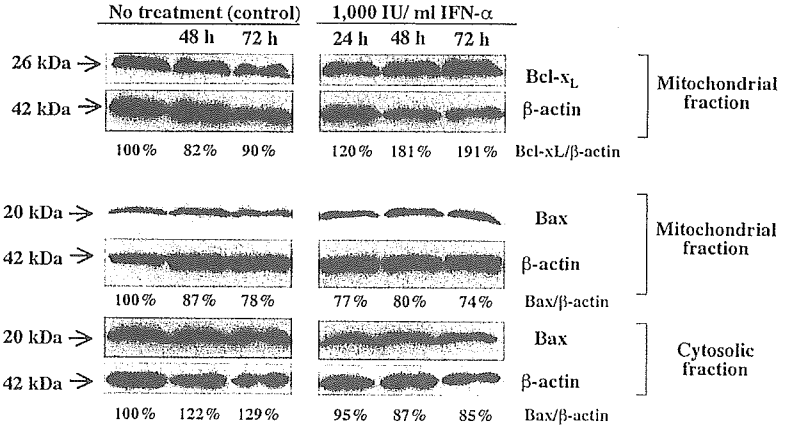

Figure 5. Effects of 1,000 IU/ml IFN-α on Bcl-x_L and Bax expression in the mitochondrial and cytosolic fractions, analyzed by Western blotting. The intensities of the bands were quantified densitometrically using the NIH Image 1.61 software program. Cytochrome c and Smac/DIABLO levels were normalized against the ß-actin level used as an internal control. The normalized levels of cytochrome c and Smac/DIABLO were comparatively analyzed.

the cytosolic fraction increased (Fig. 4). This indicates the release of these proteins from mitochondria to cytosole. The band of cytochrome oxidase subunit II (COX) was expressed in the mitochondrial fraction but not in the cytosolic fraction, which shows that the proteins in the mitochondrial fraction were not intermixed with the proteins in the cytosolic fraction.

In the mitochondrial fraction of KIM-1 cells, the expression of Bcl-x_L increased over time after the IFN-α treatment, whereas the expression of Bax slightly decreased in comparison to the no-treatment group at 24 h (77% vs. 100%) but then the expressions in the 2 groups were maintained at similar levels at the 48 and 72 h (Fig. 5). Bax in the cytosolic fraction of the no-treatment group slightly increased over time, while

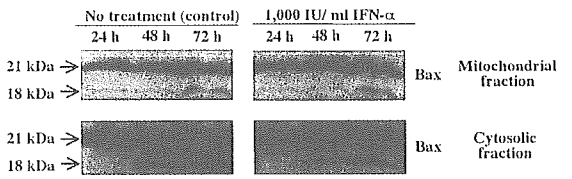

Figure 6. Effects of 1,000 IU/ml IFN-α on the expression of p18 Bax, which is the cleaved form of p21 Bax in the mitochondrial and cytosolic fractions, analyzed by Western blotting.

that of the treated group slightly decreased. Regarding Bax, expression of p18 Bax, which is the cleaved form of p21

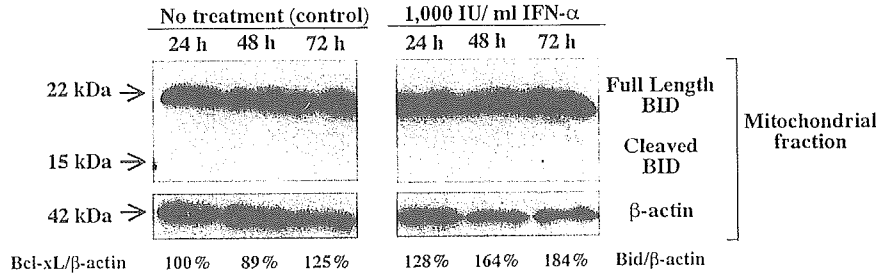

Figure 7. Effects of 1,000 IU/ml IFN-α on the expression of full-length and cleaved forms of Bid in the mitochondrial fraction, analyzed by Western blotting. Bid level was normalized against the level of ß-actin, which was used as an internal control. The normalized Bid level was comparatively analyzed.

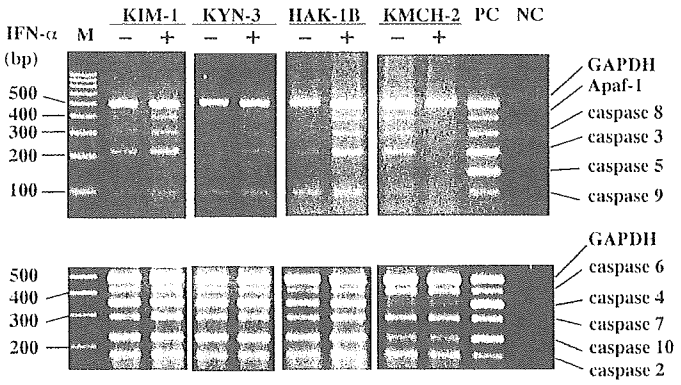

Figure 8. RT-PCR analysis on the mRNA expression of the apoptosis-related molecules in the 4 liver cancer cell lines. PCR products were electrophoresed in 4% NuSieve agarose gel and stained with ethidium bromide. A positive control (PC) for each product was provided by the manufacturer. No DNA bands were produced with identical volumes of the PCR reaction mixtures without the addition of cDNA, and they served as negative controls (NC). Lane M shows DNA molecular-weight markers.

Bax, was examined by using another antibody, but there were no differences between the no-treatment and treated groups (Fig. 6). The presence of the full-length Bid in the mitochondrial fraction was confirmed but there was no cleaved form (15 kDa band, Fig. 7). The expression of the full-length Bid in the treated group increased over time more than in the no-treatment group.

Apoptosis-related molecule mRNA expression induced by IFN-α. Expression of mRNA of 10 apoptosis-related molecules was examined in the no-treatment and treated groups at the 72 h in the 4 cell lines. In 2 IFN-α-mediated apoptosis-sensitive cells (KIM-1 and HAK-1B), the bands of caspase-3, -8 and -9, and Apaf-1 were expressed or their intensities increased. On the other hand, in the 2 resistant cell lines, KYN-3 had slight increases of band intensities for caspase-3, -8 and -9, but no other increases were observed in either cell line (Fig. 8).

Discussion

In the experiment using the broad spectrum caspase inhibitor Z-VAD-fmk, IFN-α-mediated apoptosis was completely suppressible in HAK-1B. This indicates that IFN-α-mediated apoptosis in this cell line depends on caspase. On the other hand, IFN-α-mediated apoptosis in KIM-1 was unsuppressible when the cells were treated according to the same procedures as for HAK-1B. In KIM-1, however, IFN-α-mediated apoptosis was suppressed more apparently, but incompletely by adding an inhibitor at a higher concentration before the culture and at the 24 h. This suggests 2 possibilities: a) caspase activity in KIM-1 is higher than in HAK-1B, and a higher concentration of the inhibitor is necessary to suppress their apoptosis, and b) instability of the inhibitor is present in the culture of KIM-1 cells.

Regarding the IFN-α-mediated apoptosis of KIM-1, we obtained several findings that suggest the involvement of the mitochondrial apoptotic pathway. In the protein level, there were releases of mitochondrial proteins (cytochrome c and Smac/DIABLO); activation of initiator caspase-8 and -9, and effector caspase-3, and -7; and cleavage of cellular substrate PARP. In the mRNA level, involvement of caspase-3, -8 and -9, and Apaf-1 is suggested.

Previous studies suggest that upon activation caspase-8 directly activates several caspases including caspase-3, -6, -7 and -10 as an initiator in different forms of apoptosis (11,26). In addition, caspase-8 induces Bid cleavage and the translocation of truncated cleavage product (tBit) to mitochondria where it triggers cytochrome c release (27,28). In our current study, mild increase in the expression of full-length Bid was observed over time in IFN-α-treated KIM-1 cells, but there was no clear cleavage of Bid. Therefore, release of cytochrome c through tBid was not likely to occur. However,

Desagher *et al* (29) found that, during certain types of apoptosis, full-length Bid translocates to mitochondria and binds to Bax, leading to a change in conformation of Bax and to cytochrome c release from mitochondria. Therefore, further studies are necessary to clarify whether the increase of full-length Bid expression, not the cleavage of Bid, is related to the release of cytochrome c.

In the release mechanism of cytochrome c, multiple stimuli act on mitochondria and induce the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore, and this causes $\Delta\Psi_m$ disruption and release of caspase-activating proteins such as cytochrome c (14,30). Bcl-2 and Bcl-x_L are reported to prevent PT whereas Bax is reported to induce PT and the release of cytochrome c (30,31). Expression level of Bcl-x_L in mitochondrial fraction increases with IFN-α treatment, as seen with whole cell lysate in our previous study (10). We consider that an anti-apoptotic protein Bcl-x_L level is not likely to contribute to the susceptibility of IFN-α-mediated apoptosis. Regarding Bax, a pro-apoptotic protein, various apoptotic stimuli are reported to induce its translocation from the cytosol to mitochondria (32). Juang *et al* (17) showed translocation of Bax from cytosol to mitochondria in IFN-ß-mediated apoptosis, however, Sangfelt *et al* (33), Yanase *et al* (18) and our own studies did not find such translocation in IFN-α-mediated apoptosis. Yanase *et al* (18) showed the expression of 18-kDa fragment (p18 Bax), which is a cleavage fragment of endogenous p21 Bax (21 kDa), in mitochondrial fraction, and state that modulation of endogenous p21 Bax is implicated in IFN-α-induced apoptosis. In the present study, p18 Bax expression in both the mitochondrial and cytosolic fractions was similar in the KIM-1 cells with or without IFN-α treatment. This suggests that at least in the KIM-1 cell line, p18 and p21 Bax are not essential factors for the induction of IFN-α-mediated apoptosis. A recent study of Panaretakis *et al* (20) that used a multiple myeloma cell line (U266), showed that IFN-α-induced apoptosis involves Bak and Bax activation with conformational changes via distinct mechanisms involving an unknown protease. It is necessary to examine whether similar changes occur in HCC cell lines. Smac/DIABLO is a protein that is released with cytochrome c from mitochondria during apoptosis, and this protein functions to promote caspase activation by associating with the Apaf-1 apoptosome and inhibiting apoptosis inhibiting proteins such as XIAP (34-36). Release of Smac/DIABLO from mitochondria was identified in IFN-α-mediated apoptosis of KIM-1 cells, and this would promote IFN-α-mediated apoptosis.

Although KYM-3 and KMCH-2 are resistant to IFN-α-induced apoptosis, IFN-α induces blockage of the cell-cycle at the S-phase in KYN-3, and the G_1 phase in KMCH-2 (10). Therefore, activation of Jak/signal transducer and activator of the transcription signaling pathway and certain IFN-inducible genes could be induced by IFN-α in these cells, but the cells may have abnormality in the caspase(s) and/or apoptosis-related molecules in mitochondria. Full-length caspase-9 and -8 expression levels in KMCH-2 were constitutively lower than in the other cell lines, and this would relate to the resistance. Recent studies indicate that caspase-8 expression acts as a key determinant of sensitivity for apoptosis induced by death ligands or cytotoxic drugs (37). We expect that the mechanisms of IFN-α-mediated apoptosis will become clearer by examining the expression patterns of multiple apoptosis-related genes using cDNA microarray analysis in the 4 cell lines with or without IFN-α treatment.

In conclusion, our current findings indicate the involvement of the mitochondrial apoptotic pathway with the activation of various caspases in the IFN-α-mediated apoptosis mechanism in liver cancer cells.

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第4章　急性肝炎，劇症肝炎の診断と治療

急性肝炎の診断と治療（各論）：(2) B型急性肝炎とB型慢性肝炎急性増悪

要旨

B型急性肝炎やB型慢性肝炎急性増悪は，その重症度によって，適切な治療方針を立てなければならない．抗原抗体系やウイルス量などの血清学的検査を中心に診断を行う．急性感染は多くが一過性で軽快する．急性増悪ではインターフェロンやラミブジンによる治療を行う．重症化や劇症化時には肝移植を含めての治療を考慮する．劇症化の有無を的確に判断し，早期に適当な治療を行うことが重要である．

●キーワード

B型肝炎ウイルス
B型急性肝炎
B型慢性肝炎急性増悪

はじめに

我が国におけるB型肝炎ウイルス（HBV）の感染者は人口の約1％，100万人強と考えられている．しかし，医療機関を受診しない無症候性キャリアも多いため，その正確な感染率はいまだに明らかでない．HBVによる感染は，日本ではほとんどが，母児による垂直感染である．それも，HBワクチンや抗HBs免疫グロブリン（HBIg）の開発により，予防可能となっている．しかし，不幸にも感染が成立した場合，そのほとんどはHBキャリアとなり，終生感染が持続し，自然治癒することはまれである．年齢を経るにつれて，肝機能異常（AST，ALTの上昇）が出現し，やがて慢性肝炎や肝硬変，中には肝癌を発症する．一方，成人になってからの感染は水平感染が主で，急性肝炎として一過性感染を起し，持続感染することはまれである．しかし，時に重症化や劇症化を引き起す．B型肝炎の診断については，以前から知られる抗原抗体系の血清マーカーに加え，HBV－DNAやprecore変異株やcore promoter変異株およびgenotypeの測定などが可能となり，病態の詳細な把握が可能となった．治療に関しては，近年新しい抗ウイルス薬の登場により，選択肢が広がった．そこで本稿では，前半でB型急性肝炎の診断と治療について，後半でB型慢性

肝炎急性増悪について述べたい．

B型急性肝炎の診断

ここでは，母児感染を除く，成人期以降の水平感染による初感染について述べる．今日では医療従事者の針刺し事故や血液汚染を除けば，性行為を介して感染するいわゆる性行為感染症（STD）が大部分である[1]．輸血による感染はスクリーニング検査の確立された現在はほぼ皆無だが，一部で occult HBV による感染血が検査をすり抜けて感染を起す例が報告されている．慢性化率は2～10％と言われ，高齢者や免疫状態の悪い患者に多い．潜伏期間は1～6ヵ月と幅がある．

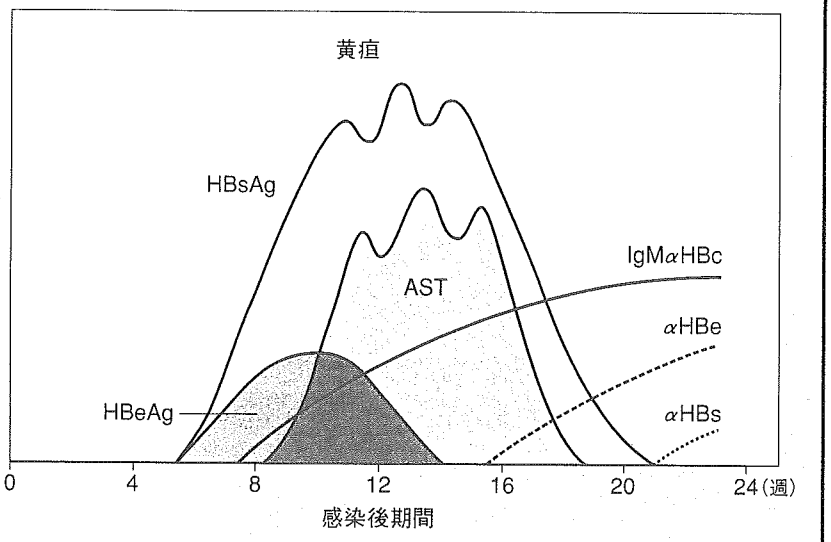
診断には血清学的検査が最も有効である．HBV の抗原抗体系にはB型肝炎ウイルス表面（HBs）抗原，HBs 抗体，B型肝炎ウイルスコア（HBc）抗原，HBc 抗体，B型肝炎ウイルスe（HBe）抗原，HBe 抗体，がある（表1）[2]．

HBs 抗原は血清トランスアミナーゼ値の上昇に先立ち，感染後約6週で血中に現れ，発病後，3ヵ月までには通常消失する（図1）．6ヵ月以上持続する場合は慢性化を考える．HBs 抗体は回復期と中和抗体を意味する．HBc 抗原は抹消血中には認められない．HBc 抗体には IgM 型と IgG 型がある．高力価 IgM 型 HBc 抗体の存在は感染初期を意味し，B型急性肝炎の診断となる．IgG 型 HBc 抗体はB型慢性肝炎もしくは既往感染を意味する．HBe 抗原の存在はウイルス産生が盛んで感染力が高いことを意味する．急性肝炎時も HBs 抗原とほぼ同時期に一過性に増加し早期に消失する．HBe 抗体は急性肝炎の回復期もしくは持続感染を意味する．急性肝炎で HBe 抗体が出現すれば，完全に回復する可能性が高い．B型急性肝炎の診断には HBs 抗原陽性，IgM 型 HBc 抗体強陽性が有用である．なお，IgM 型 HBc 抗体はB型慢性肝

表1　B型肝炎ウイルスマーカーの血清学的意義

マーカー	意義
HBs 抗原	急性あるいは慢性B型肝炎
IgM 型 HBc 抗体	急性B型肝炎（高力価） 慢性B型肝炎（低力価）
IgG 型 HBc 抗体	B型肝炎既往感染（HBs 抗原陰性） 慢性B型肝炎（HBs 抗原陽性）
HBs 抗体	B型肝炎に対する中和抗体
HBe 抗原	急性B型肝炎，続けば持続感染状態
HBe 抗体	回復期または持続感染状態
HBV−DNA	持続感染状態

図1　急性B型肝炎の経過



HBsAg：B型肝炎表面抗原，HBeAg：B型肝炎e抗原，
IgMαHBc：B型肝炎コア抗原に対するIgM抗体，
AST：アスパラギン酸アミノトランスフェラーゼ，αHBe：B型肝炎e抗原に対する抗体，
αHBs：B型肝炎表面抗原に対する抗体

炎の急性増悪時にも弱陽性を示すことがあるが，その場合は IgG 型 HBc 抗体が 200 倍希釈でも高力価であり，両者の鑑別が可能である．

血中 HBV－DNA の検出はウイルス増殖の最も鋭敏な指標であり，血清トランスアミナーゼ値ともよく相関する．血清トランスアミナーゼ値は現在の肝炎の程度を知る最も有用なマーカーとなる．多峰性に変動を繰り返す場合は慢性化に注意が必要である．

B型急性肝炎では感染の鎮静化とともに陰性化する．しかし，肝細胞内には，一過性感染後も HBV－DNA が検出され，肝炎鎮静後も肝内からは完全にウイルスが消失していないことを当院では報告している[3]．

劇症化率はB型急性肝炎全体で約2％と推定される．しかし，プロトロンビン時間が 40％ 未満のいわゆる重症型の急性肝炎では約 30％ が，肝性脳症Ⅱ度以上を合併し劇症肝炎に進展する[4]．劇症化の予測には，厚生労働省の研究班による予知式[5]と，与芝らの予知式[6]がよく用いられる（表2）．双方の式で算出される劇症化確率はしばしば異なるため，複数の式を用いて予後を判断するべきである．

表2　急性肝炎重症型の劇症化予知式

与芝の式

$\lambda = \text{logit}(P) = -0.89 + 1.74 \times$ 成因 $+ 0.056 \times$ T. Bil (mg / dl) $- 0.014 \times$ ChE (IU / L)

成因：HAV または HBV 初感染　1，その他　2

研究班の式

$\lambda = \text{logit}(P) = -2.7469 + 0.0914 \times$（年齢）$+ 0.1255 \times$ T. Bil (mg / dl) $- 0.1534 \times$ PT (%)

劇症化確率 $(p) = 1 / (1 + e^{-\lambda})$

B型急性肝炎の治療

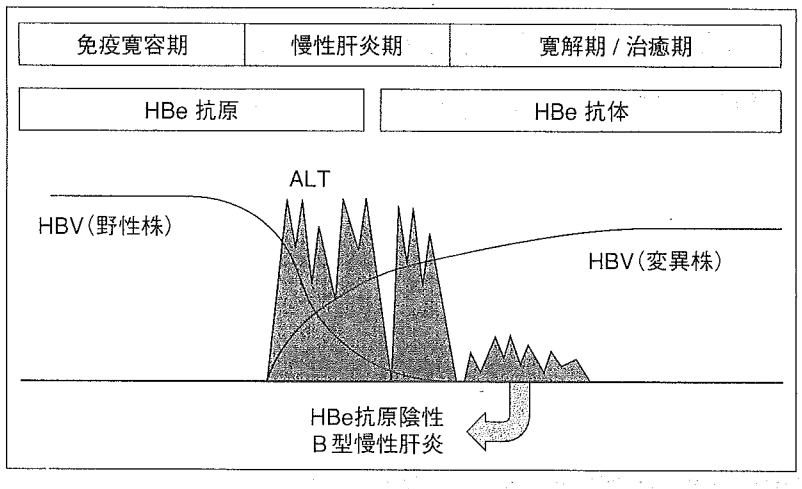
多くの場合3ヵ月以内に肝炎は鎮静化するので，入院による安静と食事療法の対処療法のみで根治可能である．食事は高タンパク，高カロリーが基本であるが，栄養過多による脂肪肝に注意が必要である．食欲低下を認める場合はビタミン剤を添加した補液が行われる．当院では全身倦怠感や食欲不振などの自覚症状の消失，血清トランスアミナーゼ値 100 IU / L 未満，血清総ビリルビン値 2.0 mg / dl 未満を退院の目処としている．

B型急性肝炎の中でも，重症化，劇症化を来す症例を数％で認める．血清総ビリルビン値やプロトロンビン時間などの肝予備能に注意しながら，劇症化の予知式[5)6)]などによる予測を行い，早めの対策が救命につながる．抗ウイルス薬のラミブジンをB型急性肝炎に使用し有効との報告[7)]もあるが，急性感染では血中のウイルスは早期に消失するため，ラミブジンがこの消失動態に影響するかは，まだ明らかなエビデンスはない．重症化，劇症化時の詳しい治療は劇症肝炎の治療の項に譲る．

B型慢性肝炎急性増悪の診断

B型慢性肝炎や血清トランスアミナーゼ正常の HB キャリアの患者が，急激な血清トランスアミナーゼ値の上昇と，時に肝機能の低下や全身倦怠感を引き起す．これを急性増悪と呼ぶ．その際に HBe 抗原陽性から HBe 抗体陽性へのセロコンバージョンを起すことがある（図2）[2)]．自然経過でのセロコンバージョンは年率 10～15％ と言われ，インターフェロン（IFN）などの抗ウイルス療法後にみられるこ

図2　HBe 抗原から HBe 抗体へのセロコンバージョン（SC）



SC は HBV（野性株）が pre-core 遺伝子あるいは core promoter 遺伝子に変異を来した HBV（変異株）となるために生じる．SC 後も HBV 増殖が持続し慢性肝炎（HBe 抗原陰性）が持続することがまれにある．

ともある．しかし，中には重症化，劇症化を来し，肝不全となる症例を認める．近年，HBe 抗原陰性でも HBV－DNA 陽性のB型慢性肝炎患者の存在が確認され，これらは HBe 抗原が産生されない pre-core 変異株や core promoter 変異株によることが報告されている[8]．HB キャリアやB型慢性肝炎からの急性増悪症例で肝不全となり死亡した症例の中には，HBV－DNA が 7.6 log copies / m*l* を超える高ウイルス症例のみならず，今述べた HBe 抗原陰性 HBe 抗体陽性の低ウイルス症例も認められる．したがって，抗原抗体系の血清マーカーのみにとらわれず，血清総ビリルビン値やプロトロンビン時間などの肝予備能を見ながら，重症化や劇症化を防ぐ必要がある．

B型慢性肝炎急性増悪の治療

急性増悪時の治療目標は ALT の正常化と重症化の防止である．基本的にはB型慢性肝炎の治療すなわち，IFN もしくはラミブジンなどの抗ウイルス薬の使用が中心となる．HB キャリアからの急性増悪例では予後の悪い亜急性型の劇症肝炎に進展する可能性が高く，肝移植も考慮しながらの診療が必要である．HBe 抗体陽性 HB キャリアに，他疾患でステロイドや免疫抑制剤を使用すると，肝炎が増悪する

表3　B型肝炎治療の新しいステージ分類

HB stage	0	I	II	III	IV	V
HBsAg	+	+	+	+	+	−**
HBeAg	+	+	+	−	−	−
HBV−DNA (copies/ml)	不問	$10^{7.6}$≦	$10^{7.6}$>	10^{5}≦	10^{5}>	不問
ALT	持続正常	持続正常以外	持続正常以外	不問	不問	不問
年齢	不問	若年 / 高年* (Ia / Ib)	若年 / 高年* (IIa / IIb)	不問	不問	不問
発癌リスク	極めて小	小 / 大	小 / 極めて大	極めて大	極めて小	極めて小
治療	不要	F_2 以上 IFN / IFN+ ラミブジン	IFN / ラミブジン	ラミブジン	不要	不要

*：若年　男性30歳未満，女性35歳未満　　高年　男性30歳以上，女性35歳以上

**：HBsAg（+）の時期が確認されていること

略語：巻末の略語集参照

ことがあり，注意が必要である．一般にウイルス量の増加が高度な症例では，肝炎も重症化する例が多いので，早期より積極的にラミブジンを使用するべきである．いずれにしても，急性感染同様に，劇症化の予知式[5)6)]などを用いて，早めの治療が必要である．

最も重要なのは，治療開始のタイミングである．特にラミブジンの開始時期にはさまざまな見解がある．プロトロンビン時間が40％をきるような重症例での早期使用は当然であるが，HBキャリアからの急性増悪と慢性肝炎からの急性増悪では異なった基準を設けるべきとの意見もある[9)]．すなわち，ラミブジンの有効性の検討結果から，HBキャリアからの急性増悪例ではプロトロンビン時間が60％未満，慢性肝炎からの急性増悪例では総ビリルビン値が5 mg / dl 以上で速やかにラミブジンを投与すべきと考えられる．

ラミブジン長期投与例においてみられる耐性株出現による breakthrough hepatitis が原因の急性増悪例では他の抗ウイルス薬の併用が試みられる．我が国では2004年12月より，アデホビルが保険適用となり，ラミブジン投与中にHBVの持続的な再増殖を伴う肝機能異常が確認された症例で，ラミブジンとの併用治療が可能となった．また，entecavir など，他の抗ウイルス薬の開発も進んでおり，今後