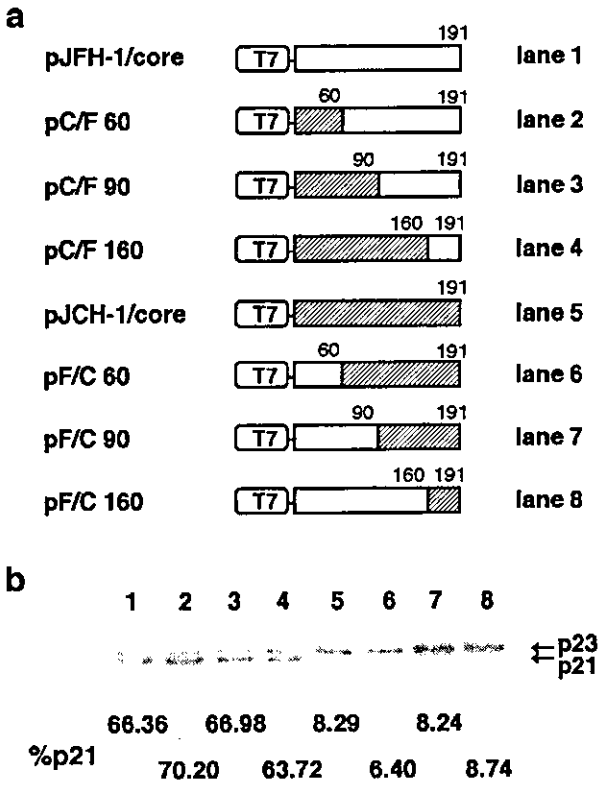

Fig. 5. Hepatitis C virus (HCV) core protein production with chimeric constructs from pJFH-1/core and pJCH-1/core. **a:** Chimeric expression vectors pC/F 60, pC/F 90, and pC/F 160 contain a sequence of clone JCH-1 in aa 1–60, aa 1–90, and aa 1–160, respectively, and a sequence of clone JFH-1in the remaining region. Similarly, chimeric expression vectors pF/C 60, pF/C 90, and pF/C 160 contain a sequence of clone JFH-1 in aa 1–60, aa 1–90, and aa 1–160, respectively, and a sequence of clone JCH-1 in the remaining region. **b:** HCV core proteins were expressed in the in vitro translation assay with canine pancreatic microsomal membrane.

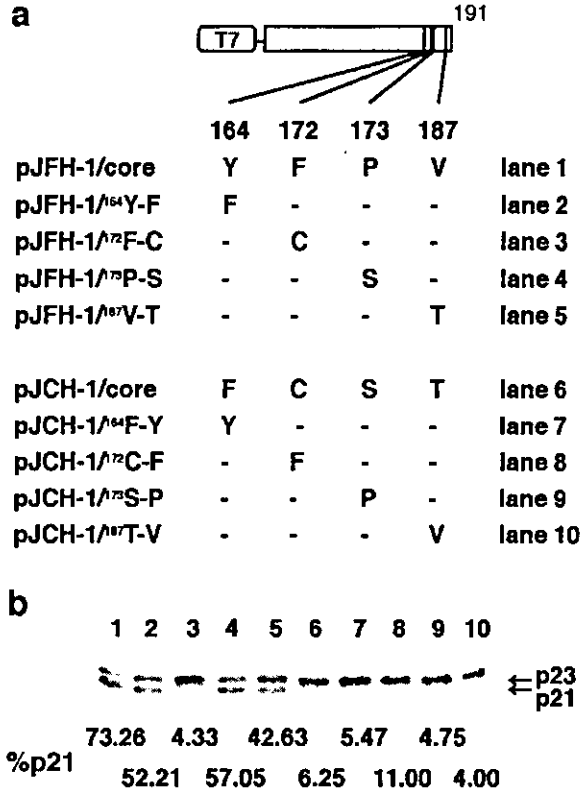

Fig. 6. Hepatitis C virus (HCV) core protein production with one mutation-introduced into constructs of clones JFH-1 and JCH-1. **a:** In these constructs, one of four amino acid residues in the C-terminus of the core region was replaced (JFH-1:JCH-1; ^{164}Y:^{164}F, ^{172}F:^{172}C, ^{173}P:^{173}S, and ^{187}V:^{187}T) and named with the replaced amino acid residue added after the original clone name. **b:** HCV core proteins were expressed in the in vitro translation assay with canine pancreatic microsomal membrane.

%p21 = 66.36%) and all pC/F constructs (Fig. 5, lanes 2–4, %p21 ranged from 63.72% to 70.20%), but was scarcely observed in the assay using pJCH-1/core (Fig. 5, lane 5, %p21 = 8.29%) and all pF/C constructs (Fig. 5, lanes 6–8, %p21 ranged from 6.40% to 8.74%). These results indicate that the principal region affecting the processing step resides in the C-terminal 31 amino acids of the core protein.

The C-terminal 31 amino acids of clones JFH-1 and JCH-1 differed by four amino acid residues (Fig. 4). To identify the roles of these four residues in p21 production, in vitro transcription and translation assays were carried out with constructs into which one to three amino acid mutations were introduced among these four amino acid residues. In the pJFH-1 constructs with one mutation (pJFH-1/^{164}Y-F, ^{172}F-C, ^{173}P-S, and ^{187}V-T), p21 core protein production was substantially decreased with pJFH-1/^{172}F-C (%p21 = 4.33%), and only slightly decreased with the other pJFH-1 constructs (%p21 ranged from 42.63% to 57.05%) (Fig. 6b, lanes 1–5). Conversely, in the pJCH-1 constructs with one mutation (pJCH-1/^{164}F-Y, ^{172}C-F, ^{173}S-P, and ^{187}T-V), no remarkable increment of p21 production was observed. In these constructs, p21 production with pJCH-1/^{172}C-F was slightly higher (%p21 = 11.00%) than pJCH-1/core (%p21 = 6.25%) or the other pJCH-1 constructs with one mutation (%p21 ranged from 4.00% to 6.25%).

To elucidate which mutations in this region of clone JCH-1 vary the p21 production from that of clone JFH-1, in vitro transcription and translation assays were undertaken with constructs into which two and three amino acid mutations were introduced among the four amino acids residues. Constructs were prepared with two amino acid mutations, ^{172}C-F and one other mutation (Fig. 7). The %p21 with these constructs were 13.65% with pJCH-1/^{164}F^{172}C-YF, 14.50% with ^{172}C^{173}S-FP, and 15.62% with ^{172}C^{187}T-FV (Fig. 7, lanes 4–6), which were slightly increased from those with pJCH-1/core (5.85%) and pJCH-1/^{172}C-F (9.37%) (Fig. 7, lanes 2 and 3). Then, constructs were prepared with three amino acid mutations in all possible combinations of these four residues (Fig. 8a). The %p21 with these

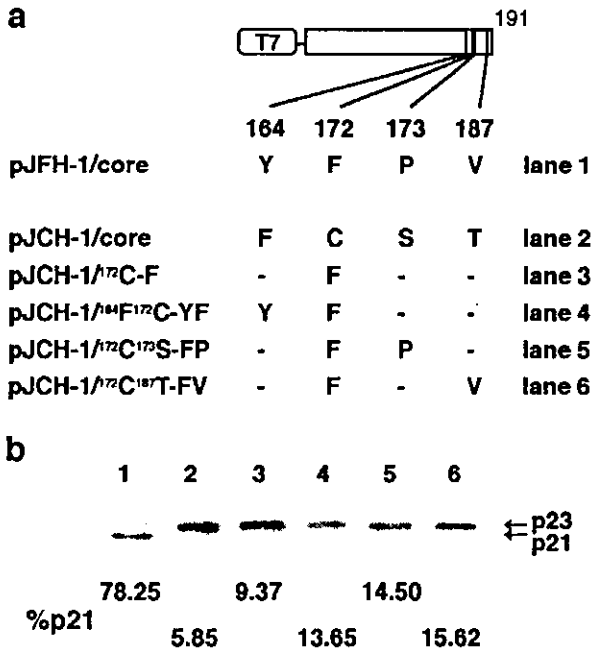

Fig. 7. Hepatitis C virus (HCV) core protein production with two mutations introduced into constructs of clone JCH-1. **a:** In these constructs, two amino acid residues, ^{172}F:^{172}C and one of the other three (^{164}Y:^{164}F, ^{173}P:^{173}S, and ^{187}V:^{187}T), were replaced. **b:** HCV core proteins were expressed in the in vitro translation assay with canine pancreatic microsomal membrane.

constructs was 46.58% with pJCH-1/^{172}C^{173}S^{187}T-FPV, 5.36% with pJCH-1/^{164}F^{173}S^{187}T-YPV, 54.25% with pJCH-1/^{164}F^{172}C^{187}T-YFV, and 27.25% with pJCH-1/^{164}F^{172}C^{173}S-YFP (Fig. 8b, lanes 3–6). Although stepwise increments of p21 production were observed in these assays with pJCH-1 constructs containing the ^{172}C-F mutation, the p21 production level with the construct pJCH-1/^{164}F^{Y73}S^{187}T-YPV was unchanged from that with the pJCH-1/core construct (Fig. 8b, lane 4). To confirm that these findings reflect of intracellular processing, the same constructs were transfected to 293T cells. In this transfection assay, the %p21 with these constructs were 41.79% with pJCH-1/^{172}C^{173}S^{187}T-FPV, 29.10% with pJCH-1/^{164}F^{173}S^{187}T-YPV, 50.50% with pJCH-1/^{164}F^{172}C^{187}T-YFV, and 41.84% with pJCH-1/^{164}F^{172}C^{173}S-YFP (Fig. 8c, lanes 3–6). From these data, the restriction for p21 processing may be more strict in the canine membrane in vitro assay than in the 293T cell assay. However, to obtain the higher p21 production level, amino acid residue ^{172}F in the core region of clone JFH-1 appeared to be essential in both in vitro and ex vivo 293T cell assay. Thus, it was found that mutant cores expressed in 293T cells to demonstrate that in vitro findings reflect sufficiently intracellular processing. Furthermore, the other three amino acid residues, ^{164}Y, ^{173}P, and ^{187}V, act in combination with ^{172}F to produce the difference in this processing step.

DISCUSSION

The core protein of HCV is encoded at the 5′ end of the long open reading frame of HCV genomic RNA [Hijikata et al., 1991]. This protein has a hydrophobic sequence at its C-terminus, and this sequence is thought to be the signal sequence of translocation for the E1 protein. Amino acid sequencing revealed that signal peptidase bound to the ER membrane cleaves the C-terminus of the core and the N-terminus of E1 proteins between aa 191 and aa 192 [Hijikata et al., 1991]. The HCV p23 core protein is membrane-bound and cleaved further at a leucine at aa 179 or aa 182 to produce the p21 protein by the recently identified eukaryotic signal peptide peptidase [Lyko et al., 1995; Hussy et al., 1996; Liu et al., 1997; Buratti et al., 1998; McLauchlan et al., 2002; Weihofen et al., 2002]. In the present study, this processing step was investigated with HCV cDNA clones isolated from patients with different clinical features. The data suggested that the p21 core protein was produced preferentially with the clone isolated from one patient with sporadic fulminant hepatitis compared with other HCV clones, and that four amino acid residues, especially ^{172}F, in the C-terminus of the core region regulated this difference in p21 core production.

Polyprotein processing is believed to be important in the regulation of gene production and replication of plus-strand RNA viruses [Hellen et al., 1989; Palmenberg, 1990; Lohmann et al., 1996]. In HCV, the mature form of core protein is produced from a precursor by host protease cleavages, and mutations at aa 173, 173 serine to methionine, and at aa 174, phenylalanine to leucine, in the C terminal hydrophobic region reportedly disrupt this processing [Liu et al., 1997]. While these sequences are highly conserved among the HCV isolates, particular substitutions were found in this region of the JFH-1 clone isolated from the patient with fulminant hepatitis [Kato et al., 2001]. Thus, these substitutions may change the tertiary structure or hydrophobicity of the precursor protein and affect the affinity or efficiency of the host protease. In fact, the substitutions of ^{172}C^{173}S in choronic hepatitis isolate to ^{172}F^{173}P in JFH-1 change the hydrophobicity. To confirm that these substitutions were not introduced artificially during the cloning procedures, cloning of multiple PCR products and direct sequencing were carried out with independently recovered cDNA from this patient, and these substitutions were definitely present in the predominant HCV clone from the patient (data not shown). The most drastic alteration of p21 production was observed by the mutation of ^{172}F in clone JFH-1 to ^{172}C in clone JCH-1. A pair of cysteines is known to make a disulfide bond in the unreduced state, such as inside the ER. Substitution of one of the paired cysteine residues may lead to structural alteration and may also affect the efficacy of protease cleavage. However, mutations of the other cysteine residues (at aa 128 and aa 184) in the core regions of clones JFH-1 and JCH-1 did not affect the production of p21 core protein (data not shown). Disulfide bonding may not be formed because most core

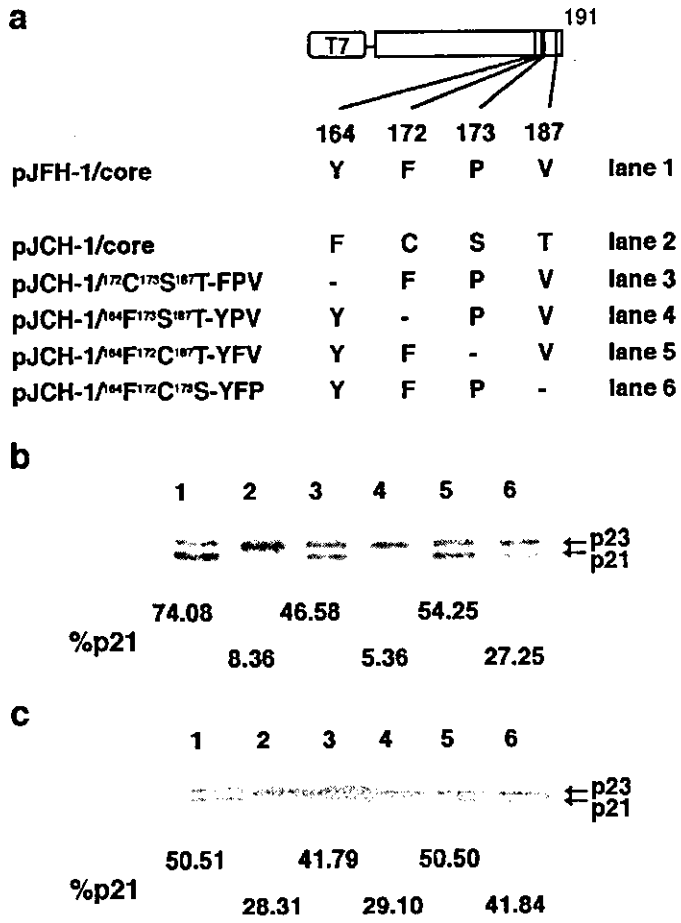

Fig. 8. Hepatitis C virus (HCV) core protein production with three mutations introduced into constructs of clone JCH-1. a: In these constructs, three of the four amino acid residues in the C-terminus of the core region were replaced (JFH-1:JCH-1; ^{164}Y:^{164}F, ^{172}F:^{172}C, ^{173}P:^{173}S, and ^{187}V:^{187}T). b: HCV core proteins were expressed in the in vitro translation assay with canine pancreatic microsomal membrane. c: The constructs were transfected to 293T cells and HCV core proteins were detected.

proteins reside within the cytoplasm, or any bonding that may form may not be very important for the structure of the cleavage site.

HCV infection usually causes a mild acute hepatitis or an asymptomatic infection, and most of the infected patients develop chronic infection. Fulminant hepatitis is a serious form of acute hepatitis, and various etiologies, especially hepatotropic viruses, are known. This disease is characterized by extensive viral replication in the host and intensive host's immune response against the virus-infected cells or tissues. In fact, some fulminant cases with HCV have been reported after withdrawal of chemotherapy or immunosuppressive drugs [Yoshiba et al., 1994; Vento et al., 1997]. In these cases, high HCV titers were associated with the onset of severe liver failure, probably due to unlimited viral replication under conditions of suppressed host immune response. On the other hand, very few sporadic cases of fulminant hepatitis with HCV have been reported [Gordon et al., 1995; Farci et al., 1996]. These facts indicate that HCV usually does not replicate vigorously. In our first diagnosed case of HCV-related fulminant hepatitis, a high titer of HCV and exceptionally severe liver damage were observed [Kato et al., 2001]. To analyze the characteristics of this virus, we sequenced the entire HCV genome and compared it with the other HCV clones from patients with chronic hepatitis. Phylogenetic analysis of these clones revealed a clustering around genotype 2a and a deviation of the fulminant clone (clone JFH-1) from the others, especially in the core, NS3, and NS5 regions [Kato et al., 2001]. In fact, clone JFH-1 demonstrated 12–16 amino acid substitutions in the core region compared with the clones isolated from patients with chronic hepatitis (Fig. 4). In the present study, a relationship between some of these substitutions and the processing of core proteins was demonstrated. Clone JFH-1 could produce the p21 core protein more efficiently than clones JCH-1–JCH-5. We also saw another fulminant hepatitis patient associated with immunosuppressive therapy, cloned HCV cDNA (clone

JFH-2), and investigated p21 core protein production. As a result, we found that clone JFH-2 did not produce p21 core protein as efficiently as clone JFH-1. These data indicated that HCV clones causing fulminant hepatitis do not always produce the p21 core protein preferentially and not always contain amino acid substitutions that affect this processing. The implications of these mutations and the altered p21 core processing phenotype for viral pathogenesis should be determined by the examination of a larger series of sporadic fulminant cases with HCV, although such cases are very rare.

In summary, processing of the HCV core protein differs among clones isolated from patients with fulminant hepatitis and chronic hepatitis, and four amino acid residues located in the C-terminus of the core region are responsible for this difference.

ACKNOWLEDGMENTS

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＜症例報告＞

ラミブジン短期投与中止後の急性増悪に対して IFN・ラミブジン併用療法が奏功したB型慢性肝炎の1例

足立　浩司　代田　博幸　柿木嘉平太
稲垣　豊　森本日出雄[1)]　小西　奎子[2)]

要　旨：症例は33歳，男性．B型慢性肝炎急性増悪にて当科へ紹介され，入院となった．AST 230 IU/l，ALT 408 IU/l，HBe抗原陽性，HBV DNAは8.3 LGE/mlと高値であったが，患者さんの強い希望でラミブジン治療を開始した．また，肝生検でF2 A1と診断された．ラミブジンを6カ月間投与したが，HBe抗原およびHBV DNA（TMA法）は持続陽性で，投与を継続するとYMDD変異株が出現する可能性が高いと考えられた．そこで，肝炎再燃時に再度抗ウイルス療法を行うこととしてラミブジン投与を意図的に中止した．中止後すぐにHBV DNA量は増加し，ALT値は4週目より上昇し始めた．ALT値が1000 IU/lを超えたところでIFNにラミブジンを短期間併用して加療したところ，ALT値が1555 IU/lまで上昇したがその後低下し，血中HBV DNAは3週目にTMA法で陰性化し，5週目にはHBe抗原からHBe抗体にseroconversionを認めた．本例は，B型慢性肝炎の急性増悪の機序や治療戦略を考える上で興味ある症例と考え報告した．

索引用語：　B型慢性肝炎　ラミブジン　IFN

はじめに

平成12年11月よりB型慢性肝炎に対して保険適応となったラミブジンはヌクレオシド誘導体の抗ウイルス剤で，副作用が少なく，抗ウイルス作用も良好であることよりその有用性が報告されている[1,2)]．しかし服薬が長期になると薬剤に耐性をもったウイルスが出現し，それが増殖することにより肝炎を起こす（breakthrough hepatitis）ことが知られている[3~5)]．一方，服薬を中止すると，押さえられていた野生株のウイルスが増殖し，肝炎を起こす（posttreatment hepatitis flare）ことがあり[6)]，治療上の問題点となっている．今回われわれは，ラミブジン投与を意図的に中止することによって生じた肝炎の急性増悪を利用して，HBe抗原からHBe抗体へのseroconversionに成功した症例を経験したので報告する．

症　例

症例：33歳，男性．

主訴：肝障害．

既往歴：10歳，虫垂切除術．

家族歴：母；B型慢性肝炎．

現病歴：2年前より検診で軽度の肝機能異常を指摘されていた．平成13年2月7日咳・痰が収まらず，近医で血液検査を受けたところ肝障害を指摘され当科へ紹介入院となった．

現症：身長165.6 cm，体重63.1 kg．結膜に貧血黄疸なし．腹部は平坦で圧痛はなく，肝を正中で3横指触知した．心肺および神経学的異常は認めなかった．

A Case of Chronic Hepatitis B Successfully Treated by the Combined Interferon-α/Lamivudine Therapy Against Acute Exacerbation Following the Cessation of the Initial Lamivudine Administration

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＜受付日2002年1月21日＞

表1　入院時検査成績

Blood chemistry		Hematology		Viral marker	
T. P.(g/dl)	6.7	WBC(/mm³)	6200	HBsAg	>2¹²
Alb.(%)	70.7	Neu.(%)	60.3	HBsAb	(−)
ZTT(u)	6.8	Eo.(%)	2.3	HBeAg	+64.6
TTT(u)	4.6	Baso.(%)	0.4	HBeAb	(−)
T. Bil(mg/dl)	0.3	Mono.(%)	5.9	HBcAb	>2¹³
AST(IU/l)	230	Lymph.(%)	31.1	IgM-HBcAb	+2.23
ALT(IU/l)	408	RBC(×10⁴)	442	HBV DNA(LGE/ml)	8.3
LDH(IU/l)	205	Hb(g/dl)	15.3	HCV-Ab	(−)
ALP(IU/l)	225	Ht(%)	45.7	Selorogical test	
γGTP(IU/l)	130	Plts.(×10⁴)	19.5	IgG(mg/dl)	1170
BUN(mg/dl)	13.5	Coagulation		IgA(mg/dl)	179
Cr(mg/dl)	0.8	PT(%)	102.3	IgM(mg/dl)	61
Na(mEq/l)	140	APTT(sec.)	29.4	ANA	(−)
K(mEq/l)	4.6	Fib(mg/dl)	225	ASMA	(−)
Cl(mEq/l)	106	HPT(%)	98.1	AMA	(−)

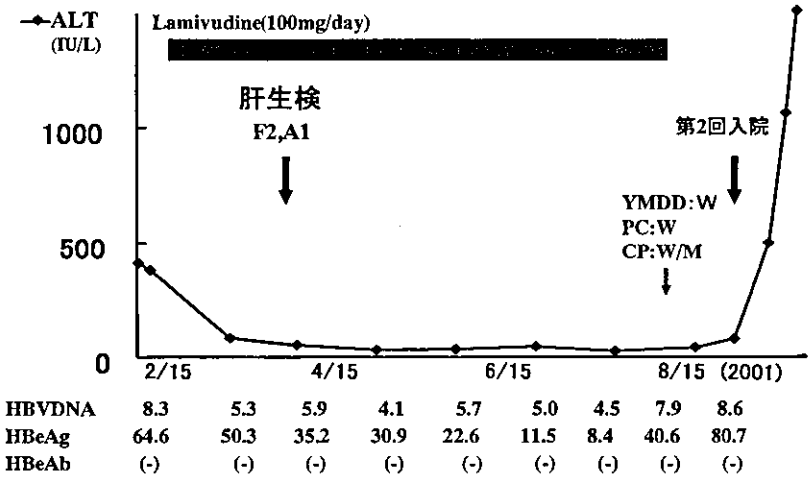
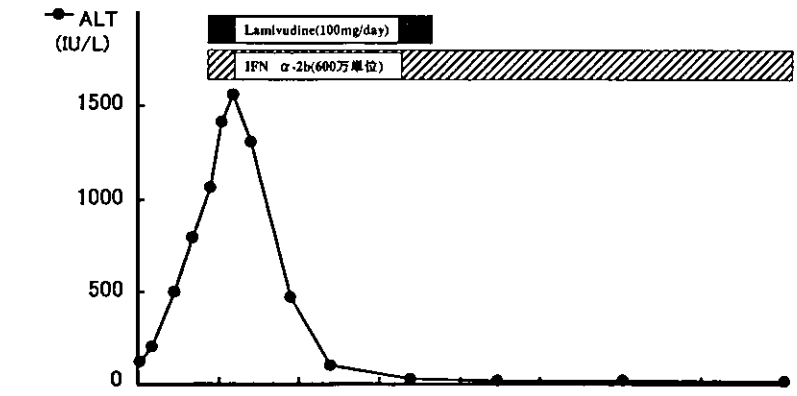


図1　初回入院後経過．W：wild, M：Mutant

入院時検査成績：AST 230 IU/l，ALT 408 IU/l とtransaminaseは高度に上昇していたが，ビリルビン値は正常で，HPT 82.4%と肝予備能も保たれていた．ウイルスマーカーでは，HBs抗原陽性，HBc抗体高力価陽性でB型肝炎ウイルスキャリアーと考えられた．また，HBe抗原陽性，HBV DNA 8.3 LGE/ml，IgM-HBc抗体陽性であり，肝機能異常はHBVによるものと考えられた．他のウイルスマーカーおよび自己抗体はすべて陰性であった(表1)．

初回入院後経過：入院3日目の肝機能検査値は，AST 172 IU/l，ALT 378 IU/l と低下し，肝炎のピークは過ぎたものと考えられた．そこでIFN療法を勧めたが，患者さんが強くラミブジンの投与を希望された．血中のHBV DNA量がTMA法で8.3 LGE/ml と多いため，耐性ウイルスが出現する可能性について説明し，入院5日目の2月20日よりラミブジン100 mg/dayの投与を開始した．その後肝機能は順調に改善し，4月2日AST 32 IU/l，ALT 51 IU/l となり，翌日退院となった(図1)．また，3月16日に肝生検を行い，CH(B)F2，A1であった．

外来通院後経過：ラミブジンを6カ月間投与し，ALT値は正常域で推移したものの，HBe抗原は陽性



	9/19	10/3	10/17	10/31	11/14	11/28	12/12	12/26	1/9(2002)
HBVDNA	8.6	8.5	4.9	<3.7	<3.7	<3.7	<3.7	<3.7	<3.7
HBeAg	80.7	85.1	12.1	1.4	(-)	(-)	(-)	(-)	(-)
HBeAb	(-)	(-)	(-)	57.3	58.4	53.9	51.1	(-)	(-)

図2　第2回入院後経過

のままで，血中HBV DNAもTMA法で陰性化しなかった．したがって，ラミブジン投与を続けると耐性株が出現する可能性が高いと判断した．その時点でウイルスはgenotype C(HBVゲノタイプEIA；特殊免疫研究所)，プレコア変異は野生型，コアプロモーター変異は混合型，YMDD変異は野生型であった．

ラミブジン投与を中止すると，野生株の増殖による肝炎の再燃が起きるが，そこで抗ウイルス療法を行うこととして，平成13年8月2日ラミブジン投与を中止した．ラミブジン中止2週後，すでに血中HBV DNAは増加し始め，6週目には8.6 LGE/m*l*となった．また，HBe抗原値も同様に増加し，ラミブジン中止によりHBVが速やかに増殖したことを示していた．ALT値は4週目40 IU/*l*，6週目79 IU/*l*と上昇は軽度であったが，HBV DNA量の増加を考慮し，9月19日第2回入院となった(図1)．

第2回入院後経過：入院時ALT値は124 IU/*l*であったが，9月28日(第10病日)には792 IU/*l*と上昇したため，10月1日(第13病日)より，IFN α-2b 600万単位(2週間連日，その後22週間週3回投与)とラミブジン100 mg/dayの投与を開始した．ALT値は投与開始4日目に1555 IU/*l*まで上昇したがその後は低下し，投与3週目には26 IU/*l*と正常値となった．血中HBV DNAは3週目にTMA法で陰性化し，5週目にはHBe抗原からHBe抗体にseroconversionを認めた．そこでラミブジンは6週間で投与中止し，IFN単独でその後の加療を行ったが，4カ月目の現在までHBVの再増殖なく，ALT値は正常域で推移している(図2)．

考　察

ラミブジンは，服薬が長期になると本剤に耐性をもったYMDD変異株の出現することが知られている[3~5]．その出現頻度は1年間で14～23％と報告され[7]，投与期間が長くなるとさらに出現率が高くなるとされている[8,9]．また，投与前の血中HBV DNA量が多い症例でYMDD変異株の出現頻度が高いことが指摘されている[5]．このYMDD変異株の増殖により肝炎を起こす"breakthrough hepatitis"が問題で，SNMCやIFNで十分対応できるとされているが[10]，死亡例も報告され[11]，その対策は確立されていない．

一方，ラミブジンの投与を中止すると，複製を抑制されていた野生株のウイルスが増殖し，肝炎が再燃(posttreatment hepatitis flare)するとされている[6]．その経過は，B型慢性肝炎に以前より行われていたステロイドリバウンド療法にみられるものと類似している．そこで，慢性肝炎の程度の軽い症例において耐性株の出現前にラミブジン投与を意図的に中止して肝炎の再燃を来し，そこで抗ウイルス療法を行う"ラミブジンリバウンド療法"の可能性が注目されている[12]．

本例は，患者さんがラミブジン治療を強く希望されたが，ラミブジン投与前の血中HBV DNAが8.3 LGE/m*l*と多く，またラミブジンを6カ月投与してもHBe抗原およびHBV DNAは陽性であり，ラミブジンを継続投与するとYMDD変異株が出現する可能性が高い症例と考えられた[5]．そこで耐性株が出現する可能性の低い6カ月[13]でラミブジンを意図的に

中止して，肝炎が再燃した時点でIFNを主体とする抗ウイルス療法を行うこととした．

ラミブジンを中止しても全例で肝炎が再燃するわけではなく，その頻度についてHonkoopらは約17%と報告している[14]．我が国の臨床試験でも，ラミブジン32週投与で15.9%，52週投与で26.9%，全体で21.1%にALT値500 IU/l以上の上昇を認めたと報告されている[15]．したがって，本例のようにラミブジン中止後にALT値1000 IU/l以上の上昇を伴う肝炎の再燃を来す症例はむしろ少ないと考えられ，いかなる症例がこのような経過を示すかを明らかにすることが重要である．

最近，Serfatyらはラミブジンと IFNの sequential投与が有効と報告した[16]．その機序として，高ウイルス量のため下がっていたHBVに対する細胞障害性T細胞活性がラミブジン投与によって回復し，IFNの効果が増した可能性を指摘している[16]．本例も最初のラミブジン投与でHBV DNA量が減少したことによりHBVに対する細胞障害性T細胞活性が回復したと推測される．その結果，ラミブジン中止後に増殖したHBVを強く排除する作用が働き，ALT値が1000 IU/lを超える肝炎再燃を来し，IFN投与がより効果的になった可能性が考えられる．

一方，肝炎再燃の程度が強すぎると劇症化する可能性も考えられる．臨床試験では242例のうち1例で死亡例を認めている．その症例は，CAH2Aの30歳代の男性で，52週間のラミブジン投与終了後YMDD野性株の増殖によってALT値が4000 IU/l以上に上昇している．劇症化に対する対策としては，まず，対象を慢性肝炎の程度の軽い症例に限定すること，次いで，肝炎再燃はYMDD野性株によることからIFNにラミブジンを併用することが重要と考えられる．さらに，肝の余力のあるところで治療を開始し，劇症化が疑われたら，ラミブジンと免疫抑制剤で加療することが必要と考えられる．本例でも，ラミブジン中止後の肝炎再燃に際し，ALT値が1000 IU/lを超えて重症化の可能性が懸念された時点で，IFNにラミブジンを併用して治療を開始し，ALT値が1555 IU/lまで上昇したが黄疸などの出現はなく，治療開始3週目でHBV DNAがTMA法で陰性化し，5週目にはHBe抗原からHBe抗体へのseroconversionとALT値の正常化を認めた．

ラミブジン短期投与中止後に肝炎再燃を来した症例は，HBVを排除する作用が強く，IFNがより効きやすい状態と推測される．したがって，症例を選択することにより，IFN治療の有効性を高めることができる可能性が考えられるが，今後本治療法の安全性も含めて，多数例での更なる検討が必要と思われる．

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Interferon alpha inhibits the nuclear factor kappa B activation triggered by X gene product of hepatitis B virus in human hepatoma cells

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Abstract X gene product of hepatitis B virus (HBV) (HBx) regulates many transcription factors including nuclear factor kappa B (NF-κB) and plays a key role in hepatocarcinogenesis. In this study, we demonstrated that the expression of full HBV genome and HBx gene similarly stimulated the transcriptional activity of NF-κB in HuH-7 human hepatoma cells, and that interferon (IFN)-α as well as dominant negative mutant of IκB kinase-α effectively inhibited the HBx-mediated NF-κB activation, but IFN-γ did not. These results suggest that IFN-α may have a function to block the NF-κB activating pathway triggered by HBx in HBV hepatocytes.

Key words: Hepatitis B virus X protein; Nuclear factor kappa B; Interferon alpha

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases worldwide, especially in several areas of Asia and Africa where hepatitis B virus (HBV) is the major etiologic factor for HCC. HBV encodes four open reading frames. Of these, the X gene product of HBV (HBx) is believed to play a key role in the hepatocarcinogenesis [1,2]. HBx is able to transactivate numerous cellular genes through activating transcription factors such as AP-1 [3,4], NF-AT [5], and nuclear factor kappa B (NF-κB) [6–8], which appears to contribute its oncogenicity.

NF-κB is involved in a number of cellular processes, including immune cell activation and development, stress responses, expression of inflammatory cytokines, and the control of apoptosis [9–13]. Recently, it has been reported that constitutive activation of NF-κB is detected in HCC tissues but not in surrounding normal tissues [14], and that NF-κB localization in HBV-related HCC was closely related to the expression of HBx [15]. These findings suggest the possible involvement of NF-κB in HCC development.

Interferon (IFN)-α plays an essential role in both antiviral and antitumor host defenses and has been used clinically for the treatment of viral infections and malignancies [16]. IFN-α treatment improves the overall clinical outcome in patients with chronic HBV infection [17,18]. IFN-α also delays or prevents HCC in patients with HBV-related cirrhosis [19]. In addition, Lai et al. [20] demonstrated a beneficial effect of IFN-α with a 31% response rate in patients with inoperable HCC caused by chronic HBV or hepatitis C virus infection. These observations assume that IFN-α may exhibit an antitumor activity in HBV-related HCC through inhibiting the HBx-mediated cellular responses.

In the present study, we aimed to clarify whether IFN-α can repress the transacting activity of NF-κB which is stimulated by transfection of either full HBV genome or HBx expressing vector in human hepatoma cells.

2. Materials and methods

2.1. Cell culture and biological reagents

The HuH-7 human hepatoma cell line was maintained in a chemically defined medium, IS-RPMI with 5% fetal bovine serum. Recombinant human IFN-α2a was provided by Nippon Roche Co. (Tokyo, Japan) and recombinant human IFN-γ1a was provided by Shionogi Co. (Osaka, Japan). In some experiments, varying concentrations of recombinant human IFN-α2a or recombinant human IFN-γ1 were added to the cell cultures.

2.2. Plasmids

The full length HBV DNA was cloned into the pGEM11Zf[+] vector (Promega, Madison, WI, USA) as described previously [21], registered in GenBank (accession number AB050018), and digested with SapI (New England BioLabs, Beverly, MA, USA) before transfection. Transfection of the full length linear monometric HBV genome can initiate a full HBV replication cycle, including production of viral RNAs, translation of viral proteins and release of virions. [21–23]. The HBx expression vector was generated from adr 125 subtype HBV DNA (JCRB Gene Bank registry no. VG024). The *NcoI–BglII* fragment of HBV DNA containing the entire coding sequence of the X gene was cloned into the expression vector, pHβApr-1, which contains the human β-actin promoter, in the sense or reverse orientation [24]. IκB kinase (IKK)α dominant negative form expression vector was kindly provided by Tularik Inc., South San Francisco, CA, USA. IκBα dominant negative form expression vector was purchased from Clontech Laboratories, Inc., Palo Alto, CA, USA.

2.3. Luciferase assay

The pNFκB-Luc (Stratagene, La Jolla, CA, USA) containing four copies of the binding sequence of NF-κB and firefly luciferase gene

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Abbreviations: IFN-α, interferon alpha; IFN-γ, interferon gamma; NF-κB, nuclear factor kappa B; IKK, IκB kinase; NIK, NF-κB-inducing kinase; HBV, hepatitis B virus; HBx, X gene product of HBV; HCC, hepatocellular carcinoma

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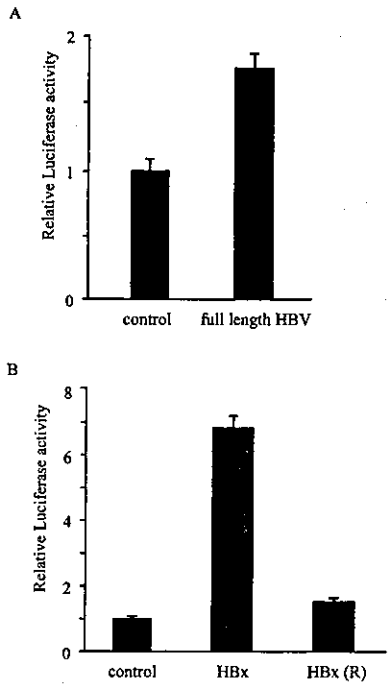

Fig. 1. Effect of HBV genome expression or HBx on the transcriptional activity of NF-κB in HuH-7 cells. 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pGEM11Zf[+] control vehicle or 0.3 μg of full length HBV DNA into HuH-7 (A). 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle, 0.3 μg of HBx expression vector or 0.3 μg of reverse-oriented HBx vector, HBx(R), into HuH-7 (B). Two days after transfection, luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

and pRL-TK-luc (Promega, Madison, WI, USA) containing the herpes simplex virus thymidine kinase promoter and expressing renilla luciferase gene were used in the assay. HuH-7 cells were grown in 24-well plates, and 0.3–0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were transfected with 0.3 μg of full length HBV DNA or pGEM11Zf[+] into the cells using FuGENE®-6 (Roche Molecular Chemicals) according to the manufacturer's protocol. Similarly, 0.3–0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc, or 0.3 μg of pLuc-MCS (Stratagene) which contains a basic promoter element (TATA box) but not NF-κB site and 50 ng of pRL-TK-luc were transfected with 0.3–0.5 μg of HBx expression vector, reverse-oriented HBx vector or pHβApr-1 into the cells. In some experiments, dominant negative IκBα expression vector or dominant negative IKKα expression vector was cotransfected into the cells. Two days after transfection, cell lysates were prepared and used for luciferase assay. The luciferase activities in the cells were determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). Data represent the ratios of firefly-luc activity derived from pNFκB-Luc or pLuc-MCS over renilla-luc activity derived from pRL-TK-luc relative to the control (pGEM11Zf[+] or pHβApr-1).

2.4. Western blot analysis

Immunoblotting with anti-HBx (Affinity Bioreagents) or anti-human β-actin (Sigma, Chemicals Co., St. Louis, MO, USA) was performed as described previously [25]. Cells were lysed by addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml PMSF (phenylmethanesulfonyl fluoride), 1 μg/ml of aprotinin, 1% NP40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg/well) was subjected to 15% SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Life Science, Buckinghamshire, UK). The enhanced chemiluminescence system (ECL; Amersham) was used for detection.

3. Results

3.1. The expression of full HBV genome as well as HBx stimulates transcriptional activity of NF-κB

The effect of full HBV genome expression on the transcriptional activity of NF-κB was determined by transient transfection assay using luciferase reporter plasmid, pNFκB-Luc, which contains four repeats of the binding sequence of NF-κB. The full length HBV DNA transfection stimulated the luciferase activities from NF-κB-Luc at a maximal value 1.8 times higher than that of the control. (Fig. 1A). Similarly, the transfection of HBx expression vector but not reverse-oriented HBx vector stimulated the luciferase activities from NF-κB-Luc at a maximal value 6.9 times higher than that of the control (Fig. 1B).

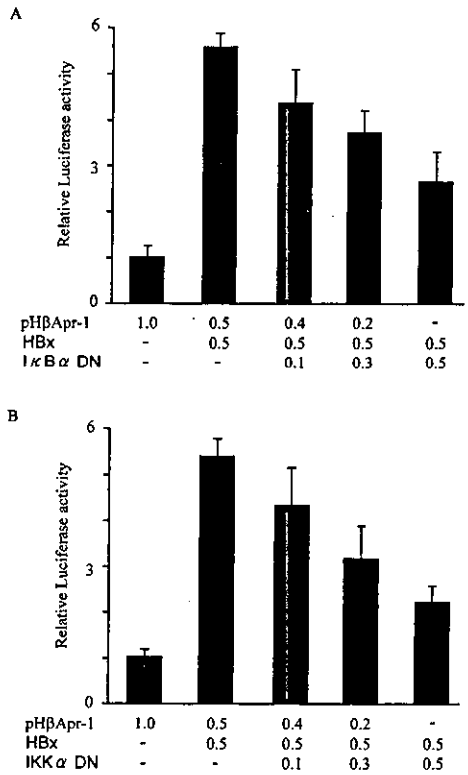

Fig. 2. Effect of dominant negative IκBα or IKKα on the HBx-mediated NF-κB activation. 0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with or without 0.5 μg of HBx expression vector into HuH-7, together with indicated amounts (μg) of dominant negative IκBα expression vector (A) or dominant negative IKKα expression vector (B). To adjust the amounts of transfected DNA, indicated amounts of pHβApr-1 were also cotransfected. Two days after transfection, luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

3.2. HBx-mediated NF-κB activation is reduced by dominant negative IKKα and IκBα

Since the stimulus-induced degradation of IκBα is essential for NF-κB activation, we examined the effect of dominant negative form of IκBα on HBx-mediated NF-κB activation. As shown in Fig. 2A, The luciferase activity from NF-κB-Luc which was stimulated by HBx was dose dependently inhibited by the transfection of IκBα dominant negative expression vector. It is well known that IKK, a multicomponent protein complex composed of IKKα, IKKβ and IKKγ phosphorylates IκB and leads its degradation. Therefore, we examined whether dominant negative mutant of IKKα inhibit HBx-mediated NF-κB activation. As shown in Fig. 2B, the luciferase activity from NF-κB-Luc which was stimulated by HBx was significantly inhibited by the transfection of IKKα dominant negative expression vector in a dose-dependent manner. Taken together, it is possible that NF-κB activation by HBx is mediated by activation of IKKα and sequential IκBα degradation.

3.3. IFN-α but not IFN-γ effectively inhibits the HBx-mediated NF-κB activation

Next, we examined the effects of IFN-α on the HBx-mediated NF-κB activation in HuH-7 cells. As shown in Fig. 3C, the luciferase activity from NF-κB-Luc which was stimulated by HBx was significantly inhibited by the addition of IFN-α

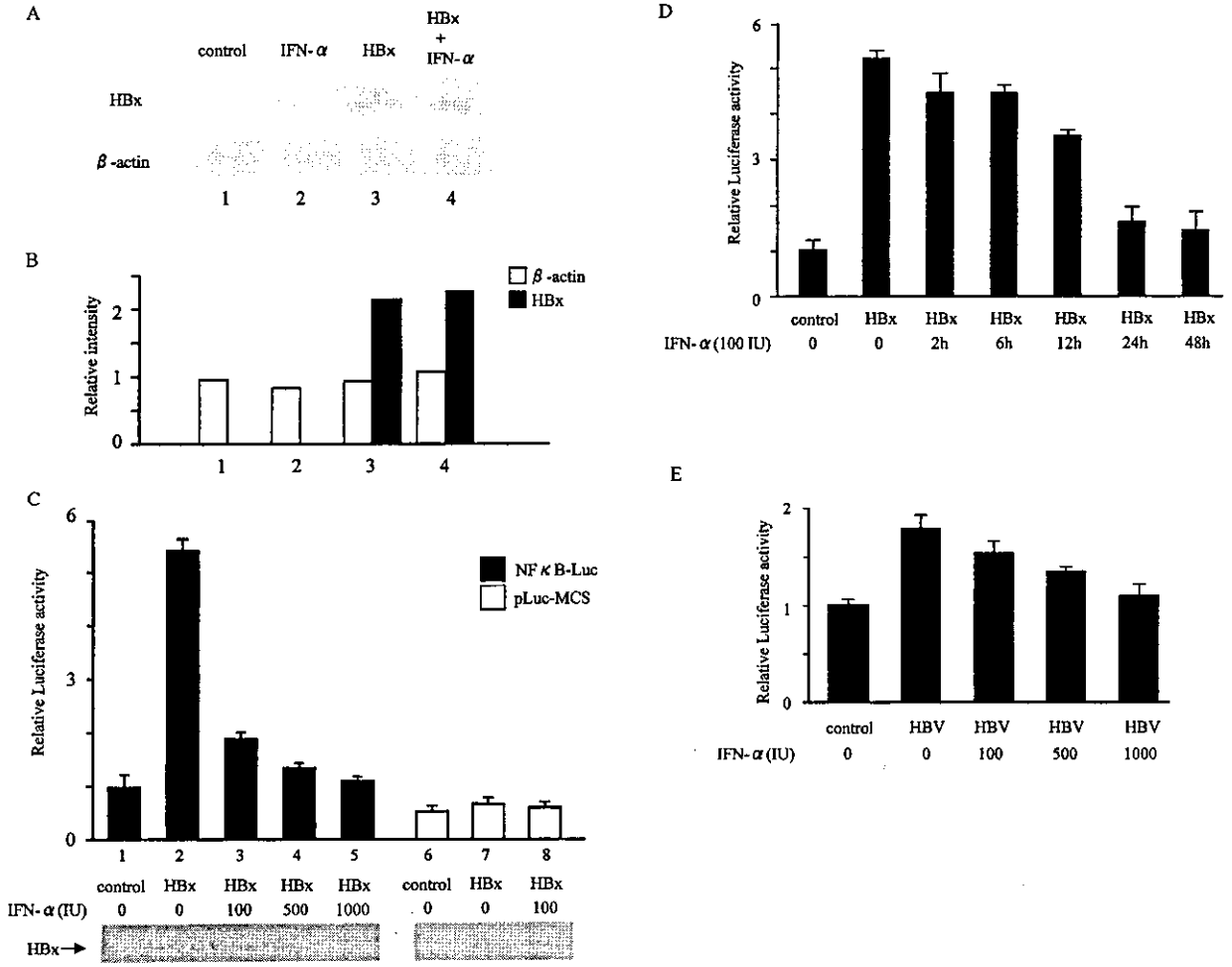

Fig. 3. Effect of IFN-α on the HBx-mediated NF-κB activation. A: HuH-7 cells were transfected with 0.3 μg of pHβApr-1 (lanes 1 and 2) or HBx expression vector (lanes 3 and 4). Twenty-four hours later, the cells were incubated with (lane 2 and 4) or without (lanes 1 and 3) 100 IU/ml of IFN-α for 24 h, then HBx expression was analyzed by Western blotting. β-actin was used as an internal control. B: The signal intensities shown in A were quantified with NIH image analysis software. Data are expressed relative to the intensity of β-actin (lane 1). The open and closed bars indicate relative intensities of β-actin and HBx, respectively. C: Combination of 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc (lanes 1–5) or combination of 0.3 μg of pLuc-MCS and 50 ng of pRL-TK-luc (lanes 6–8) were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-α for 24 h, then luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments. A representative HBx expression in each cell lysate is also shown at the bottom. D: 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with 100 IU/ml of IFN-α for varying periods (2–48 h), then luciferase activity in the cells was analyzed. E: 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pGEM11Zf[+] control vehicle or 0.3 μg of full length HBV DNA into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-α for 24 h, then luciferase activity in the cells was analyzed.

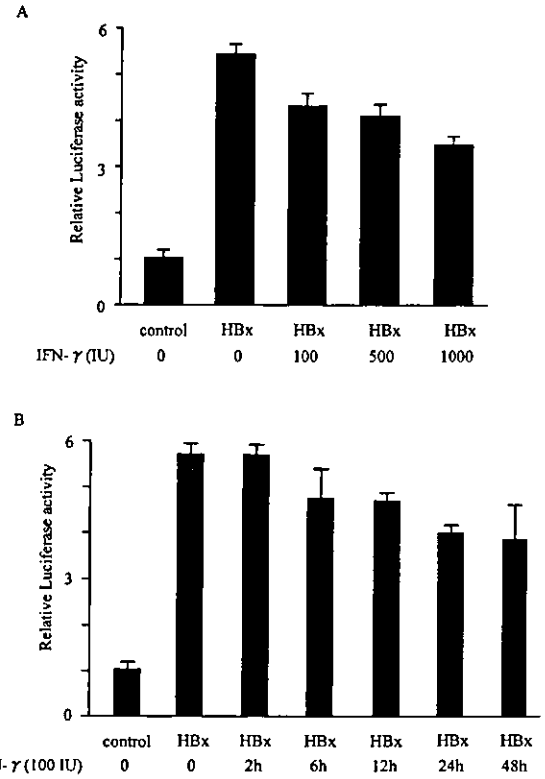

Fig. 4. Effect of IFN-γ on the HBx-mediated NF-κB activation. 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-γ for 24 h (A) or incubated with 100 IU/ml of IFN-γ for varying periods (2–48 h) (B), then luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

in a dose-dependent manner (lanes 1–5), however, these changes were not observed in the assay using pLuc-MCS reporter plasmid lacking the NF-κB binding site (lanes 6–8). In addition, inhibitory effect of IFN-α on the HBx-mediated NF-κB activation became obvious in parallel with the incubation periods (Fig. 3D). Western blot analysis showed that the IFN-α had no effect on the HBx expression in HuH-7 cells (Fig. 3A–C). Therefore, it is likely that IFN-α could block the NF-κB activating pathway triggered by HBx. Similarly, IFN-α also dose dependently repressed the NF-κB activation induced by full length HBV DNA transfection (Fig. 3E). However, in contrast to IFN-α, IFN-γ only slightly suppressed the HBx-mediated NF-κB activation even in the higher concentrations or longer incubation periods (Fig. 4A,B).

4. Discussion

In the current study, the vector-based HBx expression activated transcriptional activity of NF-κB in HuH-7 cells in accordance with other in vitro studies [6–8]. In addition, transfection of full length linear monometric HBV genome could weakly but significantly activate NF-κB, which was probably due to the very low level of HBx expression from full length HBV genome (data not shown). Recently, Guo et al. [15] reported that NF-κB was expressed diffusely in HCC tissues and its localization in the cells was closely related to the expression of HBx. They detected that NF-κB was expressed in both the cytoplasm and nuclei of HCC cells in which HBx was positive, whereas NF-κB was expressed only in the cytoplasm of HCC cells in which HBx was negative. They further confirmed that HBx could stimulate the translocation of NF-κB into nucleus in cultured HCC cells. These findings suggested that activation of NF-κB is also linked to HBx in vivo.

However, how HBx stimulates NF-κB activity has yet to be determined. HBx has been reported to activate NF-κB through turning on the ras-raf-MAP kinase cascade [6,7] and inducing the degradation of IκBα and NF-κB precursor/inhibitor p105 [7]. Alternatively, HBx could directly interact with IκBα and prevent its association with NF-κB and induce its disassociation from NF-κB [8]. In this study, we showed that HBx-mediated activation of NF-κB was repressed by expression of dominant negative IKKα and IκBα. These results are consistent with the recent report by Kim et al. [26] that dominant negative NF-κB-inducing kinase (NIK), IKK or IκB markedly attenuated the HBx-mediated NF-κB activation, and that the target disruption of tumor necrosis factor-α (TNF-α) receptor1 (TNFR1) gene completely abolished the HBx-mediated NF-κB activation. Taken together, it is likely that TNFR1 and following NIK-IKK-IκB signaling play essential roles in HBx-mediated NF-κB activation.

IFN-α are known to have antiproliferative effects on human HCC cells, both in vivo and in vitro [27,28]. The effectiveness of IFN-α in treating HCC patients has been reported positively in a few previous clinical trials [20,29]. It was also shown that IFN delays or prevents HCC in patients with HBV-related cirrhosis [19]. In addition, combination of IFN-α and 5-FU significantly prolonged the survival rate of patients with HCC [30]. These observations suggest that IFN-α has a capability of anticancer agents against HCC although its precise mechanisms are not fully understood. Recently, it was reported that IFN-α suppressed the activation of NF-κB composed of RelA-p50 and potentiated TNF-α-induced apoptosis in Jurkat cells and human cervical cancer cells [31,32]. Similarly, Shigeno et al. [33] reported that IFN-α pretreatment repressed the TNF-related apoptosis inducing ligand (TRAIL)-mediated activation of NF-κB composed of RelA-p50, and decreased its transcriptional activity in HuH-7 cells, resulting in sensitizing these cells to TRAIL-induced apoptosis. Moreover, Wen et al. [34] recently reported that overexpression of p202, an IFN-α-inducible protein, was capable of sensitizing breast cancer cells to TNF-α-mediated apoptosis through the inactivation of NF-κB by its interaction with p202. In the present study, IFN-α significantly inhibited the HBx-mediated NF-κB activation in dose- and time-dependent manner. Since HBx activates NF-κB signaling similar to TNF-α [27], it is conceivable that IFN-α can inhibit HBx-mediated NF-κB activation as well as TNF-α-mediated NF-κB activation. Whereas, IFN-γ only weakly inhibited the HBx-mediated NF-κB activation, suggesting that the IFN-α specific signaling molecules such as an IFN-stimulated gene factor-3 may mediate an inhibitory effect of IFN-α.

In conclusion, we have demonstrated that IFN-α could inhibit the HBx-mediated activation of NF-κB in human hepatoma cells. These results suggest that IFN-α may exhibit

antitumor activity in HBV-related HCC through inhibiting the NF-κB activation triggered by HBx.

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Interferon-α sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-κB inactivation

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, induces apoptosis in a variety of cancer cells with little or no effect on normal cells. Human hepatoma cells, however, are resistant to TRAIL-induced apoptosis. Since interferon-α (IFN-α) is capable of enhancing TNF-α-induced apoptosis in certain cancer cells, we evaluated the effect of IFN-α on TRAIL-induced apoptosis of human hepatoma cells. IFN-α pretreatment enhanced TRAIL-induced apoptosis of HuH-7 and Hep3B cells, in which IFN-α upregulated the expression of DR5, a death receptor of TRAIL, and downregulated the expression of survivin, which has an antiapoptotic function. In contrast, IFN-α did not enhance TRAIL-induced apoptosis of HepG2 cells, in which expression of DR5 and survivin was not affected by IFN-α. On the other hand, TRAIL activated NF-κB composed of RelA-p50 heterodimer, a key transcription factor regulating cell survival, in HuH-7 and HepG2 cells. However, IFN-α pretreatment repressed the TRAIL-mediated activation of NF-κB and decreased its transcriptional activity in HuH-7 but not in HepG2 cells. Moreover, IFN-α pretreatment clearly augmented TRAIL-mediated caspase-8 activation in HuH-7 cells. Our results suggest that IFN-α could sensitize certain human hepatoma cells to TRAIL-induced apoptosis by stimulating its death signaling and by repressing the survival function in these cells.
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Keywords: TRAIL; interferon-α, hepatoma; DR5 survivin; NF-κB

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a novel member of the TNF superfamily, is a promising candidate for cancer therapy since it preferentially induces apoptosis in a variety of cancer cells with little or no effect on normal cells (Sheridan *et al.*, 1997; Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). TRAIL triggers apoptosis through interaction with death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (Pan *et al.*, 1997; Schneider *et al.*, 1997), whereas TRAIL-R3, TRAIL-R4 and osteoprotegerin serve as decoy receptors to block TRAIL-mediated apoptosis (Degli-Esposti *et al.*, 1997a, b; Emery *et al.*, 1998). DR4 and DR5 do not only signal apoptosis through Fas-associated death domain (FADD) and caspase-8 (Kischkel *et al.*, 2000), but also activate nuclear factor-kappaB (NF-κB), which regulates the expression of survival factors such as members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP) and Bcl-xL (Wang *et al.*, 1998; Chen *et al.*, 2000). These findings suggest the existence of several physiological mechanisms that protect normal cells against TRAIL-mediated apoptosis. Indeed, administration of recombinant TRAIL to experimental animals, including mice and primates, induced significant tumor regression without systemic toxicity (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999; Chinnaiyan *et al.*, 2000). In addition, a recent study revealed that recombinant TRAIL, trimerized through the endogenous cysteine residue at position 230, does not induce apoptosis of normal human hepatocytes (Lawrence *et al.*, 2001), which have been reported to be susceptible to the other type of recombinant TRAIL (Jo *et al.*, 2000). On the other hand, high concentrations of TRAIL are necessary to induce apoptosis of certain cancer cells, including human hepatoma cells (Yamanaka *et al.*, 2000). Thus, combinations of TRAIL with several chemotherapeutic agents or radiation have been evaluated for rendering such cells susceptible to TRAIL, and some combinations have successfully enhanced TRAIL-mediated cancer cell death (Chinnaiyan *et al.*, 2000; Gibson *et al.*, 2000; Nagane *et al.*, 2000; Sun *et al.*, 2000; Yamanaka *et al.*, 2000; Zhou *et al.*, 2000; Belka *et al.*, 2001; Di Pietro *et al.*, 2001; Lacour *et al.*, 2001; Nimmanapalli *et al.*, 2001).

Interferon (IFN)-α/β plays an essential role in both antiviral and antitumor host defenses and has been used

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clinically for the treatment of viral infections and malignancies (Gutterman, 1994). Several investigators have recently reported that IFN-α/β stimulates the expression of TRAIL in T cells or dendritic cells, resulting in upregulation of their cytotoxic activities (Fanger *et al*., 1999; Kayagaki *et al*., 1999), and that the apoptosis observed in several cancer cells susceptible to IFN-α/β is mediated by induction of endogenous TRAIL in these cells (Chawla-Sarkar *et al*., 2001; Chen *et al*., 2001; Toomey *et al*., 2001). Taken together, these findings suggest that IFN-α/β may, at least in part, exhibit its antitumor activity through induction of TRAIL. Conversely, IFN-α/β has been reported to sensitize human cervical cancer cells or Jurkat lymphoma cells to TNF-α-induced apoptosis through inhibition of NF-κB activation (Manna *et al*., 2000; Suk *et al*., 2001). Since both TRAIL and TNF-α trigger death signaling and activate NF-κB (Yamanaka *et al*., 2000; Ravi *et al*., 2001), we hypothesized that IFN-α/β could sensitize cancer cells to TRAIL-induced apoptosis. In the present study, we investigated whether IFN-α pretreatment could augment TRAIL-induced apoptosis of human hepatoma cells that exhibit resistance to TRAIL. In addition, we evaluated the effect of IFN-α on TRAIL-mediated activation of both caspase-8 and NF-κB, and also analysed whether IFN-α regulated the expression of TRAIL, DR4, DR5 and apoptosis-related proteins in these cells.

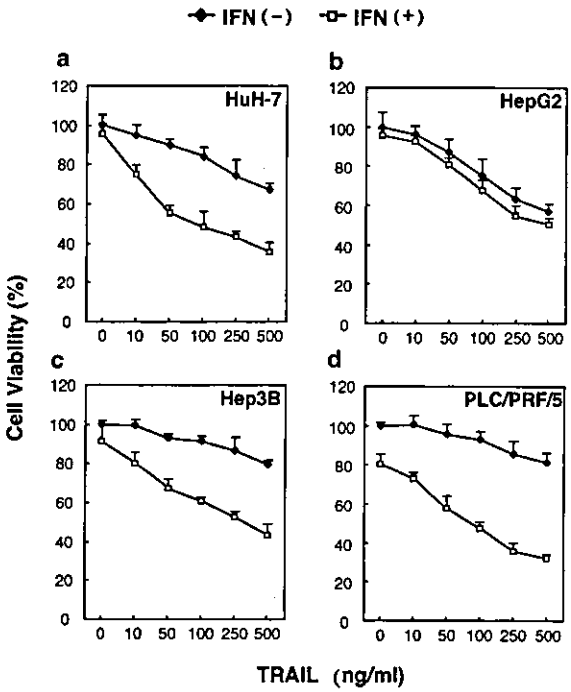

Figure 1 Effects of IFN-α on TRAIL-induced cytotoxicity in human hepatoma cells. (a) HuH-7, (b) HepG2, (c) Hep3B and (d) PLC/PRF/5 cells were preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with varying concentrations of TRAIL for 24 h. Cell viability was then determined by the colorimetric method. Results are expressed as a percentage of the control. Data represent the mean±s.d. values of the four experiments

Results

IFN-α pretreatment sensitizes human hepatoma cells to TRAIL-induced apoptosis

To elucidate the effect of IFN-α on TRAIL-induced apoptosis of human hepatoma cells, HuH-7, HepG2, Hep3B and PLC/PRF/5 were preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with varying concentrations of TRAIL for 24 h. As shown in Figure 1, TRAIL dose-dependently reduced cell viability of HuH-7 and HepG2, but higher concentrations of TRAIL were required to elicit significant cytotoxicity. Hep3B and PLC/PRF/5 were much more resistant to TRAIL-induced cytotoxicity. In contrast, preincubation with IFN-α enhanced the TRAIL-induced cytotoxicity in HuH-7, Hep3B and PLC/PRF/5, but not in HepG2, although IFN-α alone could reduce cell viability in PLC/PRF/5. Next, to examine whether IFN-α pretreatment actually enhanced TRAIL-induced apoptosis of HuH-7 and Hep3B, we examined the content of hypodiploid DNA in those cells. IFN-α pretreatment enhanced TRAIL-induced apoptosis of HuH-7 and Hep3B (Figure 2). However, such enhancement was not observed in HepG2 cells. These results indicated that IFN-α sensitized HuH-7 and Hep3B, but not HepG2 cells, to TRAIL-induced apoptosis.

IFN-α upregulates DR5 expression and downregulates survivin expression in HuH-7 and Hep3B, but not in HepG2

Recent studies have shown that expression of death receptors, DR4 or DR5, is upregulated in cancer cells by chemotherapeutic agents or by radiation, resulting in enhancement of TRAIL-induced apoptosis of these cells (Gibson *et al*., 2000; Nagane *et al*., 2000; Sun *et al*., 2000; Zhou *et al*., 2000; Di Pietro *et al*., 2001; Lacour *et al*., 2001; Nimmanapalli *et al*., 2001). We therefore examined the effect of IFN-α on expression of DR4 and DR5, as well as the expression of several apoptosis-related proteins, including procaspase 8, FLICE-inhibitory protein (FLIP), CIAP2, XIAP, survivin, Bcl-xL and Bax, by Western blotting. As shown in Figure 3, these proteins were constitutively expressed in HuH-7, HepG2 and Hep3B cells. DR5 expression was upregulated by IFN-α in HuH-7 and Hep3B but not in HepG2 cells. On the contrary, the expression of survivin, a member of the IAP family that has an antiapoptotic function, was repressed by IFN-α in HuH-7 and Hep3B but not in HepG2 cells. These results appeared to be parallel to the effect of IFN-α on TRAIL-induced apoptosis in these cells. Conversely, the expression of procaspase 8, FLIP and XIAP was almost unchanged, irrespective of the presence of IFN-α. DR4 expression

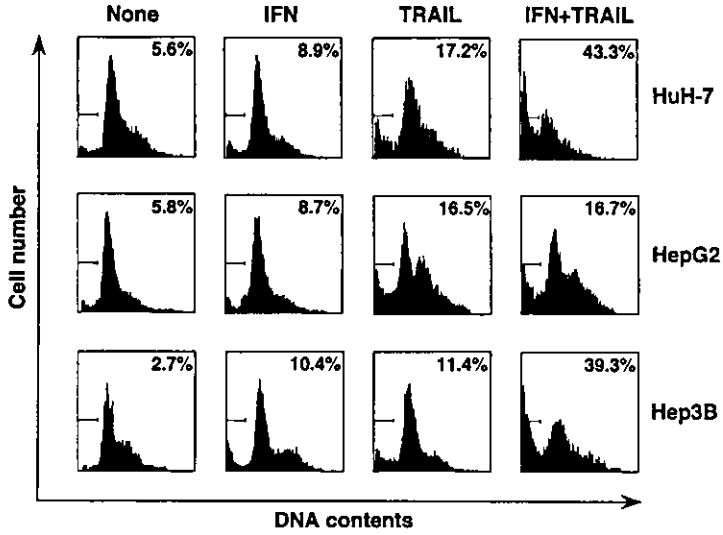

Figure 2 Effect of IFN-α on TRAIL-induced apoptosis of human hepatoma cells. HuH-7, HepG2 and Hep3B cells were preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with or without 100 ng/ml of TRAIL for 24 h. Cells were then stained with propidium iodide and subjected to DNA content analysis by flow cytometry. The percentages of cells with hypodiploid DNA are indicated. Results shown are from one representative experiment from a total of four performed

was only slightly stimulated by IFN-α in HuH-7 but not in other cells. CIAP2 expression was clearly repressed in Hep3B and slightly repressed in HuH-7 but not in HepG2 cells. The effects of IFN-α on expression of Bcl-xL and Bax differed among these cells, and even between HuH-7 and Hep3B, in which IFN-α equally enhanced TRAIL-induced apoptosis (Figure 2). DR5 and DR4 expression on the cell membrane was also analysed by flow cytometry. As shown in Figure 4, IFN-α upregulated the surface expression of DR5 in HuH-7 and Hep3B but not in HepG2 cells. On the other hand, the surface expression of DR4 was slightly stimulated by IFN-α in HuH-7 but not in other cells. These results were almost similar to those of Western blotting. We next examined the effect of IFN-α on mRNA levels of TRAIL, DR4, DR5, survivin and several apoptosis-related genes in HuH-7 cells by RNase protection assay (Figure 5). IFN-α clearly upregulated the transcripts of DR5, and weakly stimulated the transcripts of DR4 and TRAIL, which appeared as faint bands (Figure 5a). Procaspase 8 and CIAP2 mRNAs were also upregulated by IFN-α (Figure 5a and b) although this was not detectable by Western blotting (Figure 3). In contrast, survivin mRNA expression was repressed by IFN-α (Figure 5b).

To clarify the functional role of DR5 in enhancement of TRAIL-mediated apoptosis by IFN-α, we examined the effect of DR5-specific blocking chimera antibody on TRAIL/ IFN-α-induced apoptosis and also checked the impact of IFN-α on apoptosis triggered by DR5-specific agonistic antibody in HuH-7 cells. Addition of DR5-specific blocking chimera antibody dose-dependently inhibited TRAIL/ IFN-α-induced apoptosis of HuH-7 cells (Figure 6a). In addition, IFN-α sensitized the cells to apoptosis induced by DR5-specific agonistic antibody (Figure 6b) as well as to apoptosis induced by TRAIL (Figure 1). These results suggested that up-regulation of DR5 by IFN-α could contribute to enhanced TRAIL sensitivity in HuH-7 cells. Next, we also examined the involvement of survivin in resistance against TRAIL-mediated apoptosis of HuH-7 cells. In comparison with the vehicle vector transfection, transfection of the survivin expression vector which produced the flag/survivin fusion protein (Figure 7a) partially but dose-dependently inhibited TRAIL/IFN-α-induced apoptosis of HuH-7 cells (Figure 7b). These results suggested that survivin might play a role in resistance against TRAIL-mediated apoptosis of HuH-7 cells.

IFN-α inhibits TRAIL-mediated NF-κB activation in HuH-7 but not in HepG2

Since TRAIL can both trigger death signaling and activate NF-κB, thus regulating the expression of several factors involved in cell survival, we examined the effect of IFN-α on TRAIL-mediated transcriptional activity of NF-κB in HuH-7 and HepG2 cells by reporter gene transfection assay. TRAIL dose-dependently stimulated luciferase activity from the plasmid pNFκB-luc (containing four repeats of NF-κB binding sequences) in both cells. However, IFN-α pretreatment suppressed this induction in HuH-7 but not in HepG2 cells (Figure 8). We then used EMSA to determine whether IFN-α could inhibit the TRAIL-mediated NF-κB binding activity to oligonucleotides containing a NF-κB binding (κB) sequence. Nuclear extracts from unstimulated HuH-7 cells formed a complex band with the oligonucleotide probe (Figure 9a, lane 1). Addition

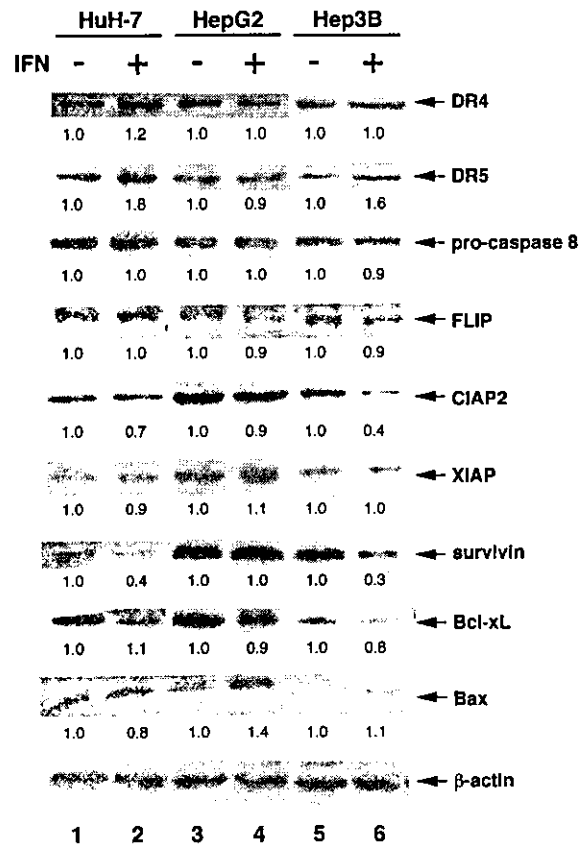

Figure 3 Effect of IFN-α on the expression of DR4, DR5 and several apoptosis-related proteins in human hepatoma cells. HuH-7, HepG2 and Hep3B cells were incubated with or without 1000 IU/ml of IFN-α for 48 h, and the expression of DR4, DR5, pro-caspase 8, FLIP, CIAP2, XIAP, survivin, Bcl-xL, Bax and β-actin in the cells was analysed by Western blotting using the appropriate antibodies. Results shown are from one representative experiment from a total of four performed. The density of each band was quantified with NIH image analysis software using β-actin as reference. Data are expressed relative to the density of control; without IFN-α in each cell (lanes 1, 3 and 5)

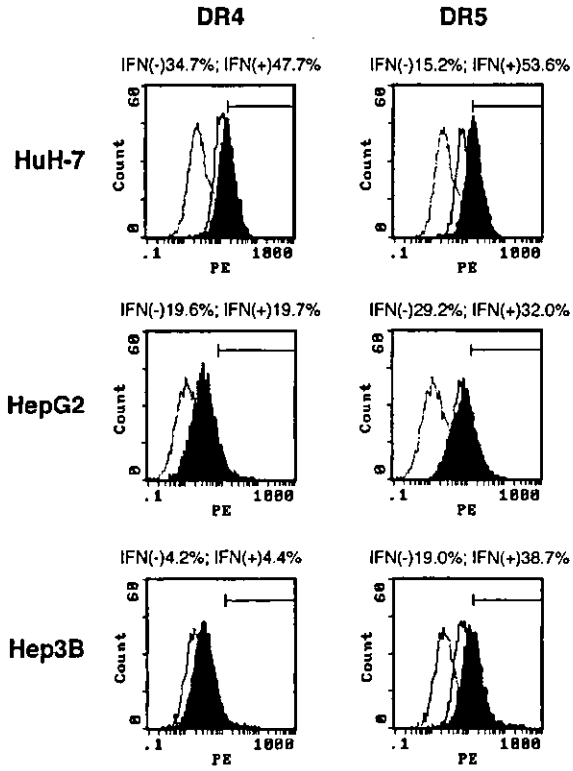

Figure 4 Flow cytometric analysis of DR4 and DR5 expression in human hepatoma cells. HuH-7, HepG2 and Hep3B cells were incubated with or without 1000 IU/ml of IFN-α for 48 h, and membrane expression of DR4 and DR5 was analysed by FACScan. The horizontal and vertical bars indicate the fluorescence intensity and the relative number of cells, respectively. Shadowed histograms represent IFN-α-treated cells, and clear histograms represent untreated cells. Histograms with gray lines represent negative controls. The percentages of positive cells are indicated. Results shown are from one representative experiment from a total of three performed

of a 100-times molar excess of the nonlabeled NF-κB oligonucleotide reduced the density of the complex band (Figure 9a, lane 5), but addition of nonlabeled AP-1 oligonucleotide did not (Figure 9a, lane 6). Moreover, addition of anti-p50 but not anti-c-Rel or anti-RelA led to a supershift of the band (Figure 9a, lanes 2–4), suggesting that NF-κB composed of p50 homodimer was interacting with the κB sequence under nonstimulated conditions. Nuclear extract from HuH-7 cells treated with TRAIL for 2 h formed an additional slowly migrating band (Figure 9a, lane 7 and Figure 6b, lane 3), which was reduced by addition of nonlabeled NF-κB oligonucleotide (Figure 9a, lane 11) and led to a supershift by addition of both anti-p50 and anti-RelA but not by addition of anti-c-Rel (Figure 9a, lanes 8–10). In contrast, nuclear extract from HuH-7 cells preincubated with IFN-α for 48 h, followed by TRAIL treatment for 2 h did not form such a slowly migrating band (Figure 9b, lane 4). These results indicated that TRAIL activated the NF-κB composed of RelA-p50 heterodimer in HuH-7, which was inhibited by IFN-α pretreatment. Such a slowly migrating band was also formed in HepG2 cells treated by TRAIL (which was not inhibited by IFN-α pretreatment) (Figure 9b, lanes 7 and 8), although similar supershifts were detected by addition of anti-p50 or anti-RelA (data not shown).

IFN-α enhances TRAIL-induced caspase-8 activity in HuH-7

Finally, we examined the effect of IFN-α on the TRAIL-mediated caspase-8 activity of cleaving IETD-pNA-conjugated substrate. TRAIL time-dependently induced caspase-8 activity in HuH-7 cells. On the other hand, IFN-α pretreatment clearly enhanced its activity, which reached a peak level 4 hour after addition of TRAIL (Figure 10).

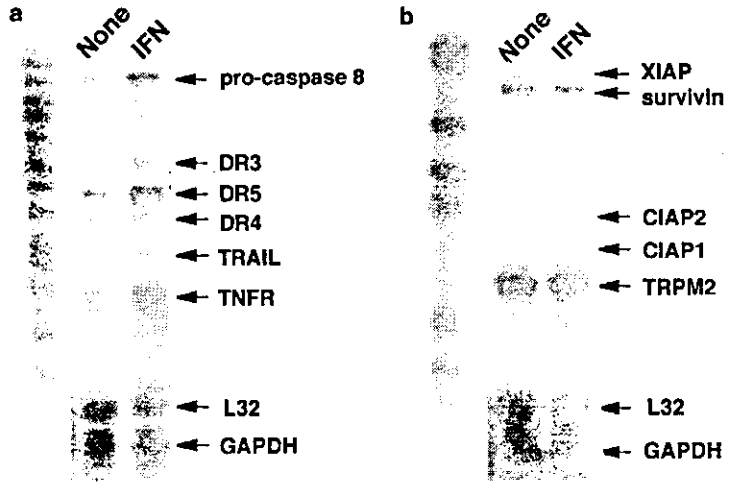

Figure 5 Effect of IFN-α on mRNA levels of DR4, DR5, TRAIL, pro-caspase 8 and IAP family genes in HuH-7 cells. HuH-7 cells were incubated with or without 1000 IU/ml of IFN-α for 48 h. Total RNA (10 μg) from the cells was subjected to analysis for mRNA levels of DR4, DR5, TRAIL, procaspase 8 and IAP family genes by RNase protection assay using human apoptosis multiprobe template sets, hAPO3-c (A) and hAPO-5c (B), respectively. Results shown are from one representative experiment from a total of three performed

Discussion

In this study, we have demonstrated that IFN-α pretreatment sensitized HuH-7 and Hep3B, but not HepG2, cells to TRAIL-induced apoptosis, and that IFN-α upregulated the expression of DR5 and downregulated the expression of survivin in HuH7 and Hep3B, but not in HepG2 cells. Recent studies have indicated that upregulation of DR5 by chemotherapeutic agents or radiation sensitizes many cancer cells to TRAIL-induced apoptosis through activation of death signaling (Gibson *et al*., 2000; Nagane *et al*., 2000; Sun *et al*., 2000; Zhou *et al*., 2000; Lacour *et al*., 2001; Nimmanapalli *et al*., 2001). In addition, we have shown that DR5-specific blocking chimera antibody inhibited TRAIL/ IFN-α-induced apoptosis in HuH-7 cells, and that IFN-α could sensitize the cells to apoptosis induced by DR5 specific agonistic antibody. Taken together, it seems that upregulation of DR5 by IFN-α enhances TRAIL sensitivity. Survivin, which is expressed in cancer tissues including hepatocellular carcinoma but not in normal tissues, represses the activities of caspases (Tamm *et al*., 1998; Ito *et al*., 2000), resulting in resistance of cancer cells to Fas- or chemotherapeutic agent-mediated apoptosis (Li *et al*., 1998; Tamm *et al*., 1998). In addition, inhibition of survivin expression, by transduction with antisense oligonucleotides or a dominant-negative expression vehicle, sensitized cancer cells to chemotherapeutic agent-mediated apoptosis (Olie *et al*., 2000; Grossman *et al*., 2001). Recently, Griffith *et al*. (2002) reported that the cellular level of survivin is closely relevant to the resistance against TRAIL-mediated apoptosis of renal cell carcinoma cells. We also demonstrated that ectopic expression of survivin repressed TRAIL/IFN-α-induced apoptosis of HuH-7 cells. These findings suggest that downregulation of survivin by IFN-α may, at least in part, account for the enhancement of TRAIL-mediated apoptosis by IFN-α. In contrast, several investigators have demonstrated that IFN-α/β stimulated TRAIL expression in certain cancer cells (Toomey *et al*., 2001; Chen *et al*., 2001), which could be involved in the antitumor activities of IFN-α. Chawla-Sarkar *et al*. (2001) reported that IFN-β, rather than IFN-α, induced TRAIL expression in melanoma cells. We also showed that IFN-α weakly stimulated TRAIL gene expression in HuH-7 cells, which may not, however, be the major cause of the enhancement of TRAIL-induced apoptosis by IFN-α because IFN-α alone could not induce significant apoptosis of HuH-7 cells (Figures 1 and 2).

The NF-κB transcription factor family consists of several structurally related proteins such as c-Rel, RelA, RelB, p50/p105, and p52/p100, which form homo- or heterodimers with each other and regulate the expression of a number of genes (Barkett and Gilmore, 1999). In the present study, nuclear extracts from HuH-7 and HepG2 cells exhibited binding activity of NF-κB composed of a p50 homodimer without any stimulation, as reported in other types of cells (Inan *et al*., 2000). TRAIL induced additional binding activity of NF-κB comprising a RelA-p50 heterodimer in these cells. Ravi *et al*. (2001) have recently reported that $RelA^{-/-}$ mouse fibroblasts are highly sensitive to TRAIL-induced apoptosis, and that anti-CD40-mediated activation of NF-κB, including RelA, effectively blocked TRAIL-induced apoptosis. Therefore, NF-κB composed of RelA-p50 appears to play a key role in the resistance of HuH-7 and HepG2 cells to TRAIL-induced apoptosis. Indeed, IFN-α pretreatment inhibited TRAIL-mediated activation of RelA-p50 NF-κB in HuH-7 cells that were sensitized to TRAIL-induced apoptosis by IFN-α. In contrast, IFN-α pretreatment could not inhibit activated HepG2 cells that