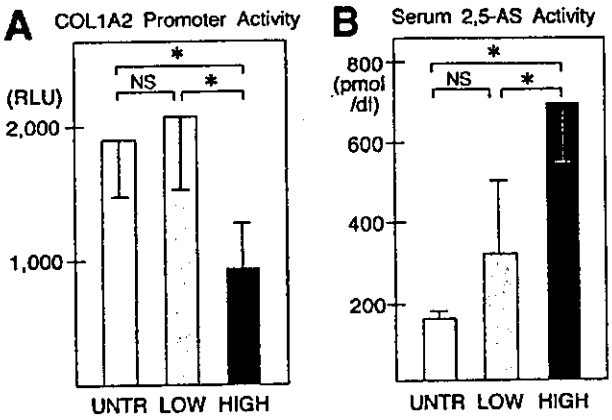

Fig. 2. Dose-dependent effects of IFN-α treatment on COL1A2 promoter activity following carbon tetrachloride administration. The transgenic mice were injected intraperitoneally with 0.1-mL/kg body weight of CCl_4, then treated with either 5,000 units/d (LOW) or 50,000 units/d (HIGH) of murine IFN-α or control saline (UNTR). The mice were killed 72 hours after CCl_4 injection and subjected to (A) luciferase assays determining COL1A2 promoter activity and (B) measurement of serum 2', 5'-oligoadenylate synthetase (2,5-AS) activity. Luciferase activity was normalized against the protein concentration of tissue homogenates. The values are mean ± SD obtained from 7 or 8 mice in each group and expressed relative to those in the control mice without CCl_4 injection shown in Fig. 1. The **asterisk** indicates that the difference between the groups is significant.

IFN-α Represses COL1A2 Expression in Activated HSC. To explore the molecular mechanisms responsible for the inhibitory action of IFN-α on COL1A2 promoter activation *in vivo*, primary cultures of rat HSC were treated with rat IFN-α or IFN-γ, and the steady-state levels of COL1A2 mRNA were determined by real-time RT-PCR. At day 3 after plating, quiescent HSC with fat droplets in the cytoplasm contained only a limited amount of COL1A2 mRNA, which was not affected by IFN treatment (Fig. 4A). Along with the period of culture on plastic dishes, HSC lost fat droplets and exhibited the phenotype of activated myofibroblasts (data not shown). At day 7, the cells expressed an approximately 40 times larger amount of COL1A2 mRNA than did quiescent cells, and the increased COL1A2 mRNA expression was significantly suppressed by IFN-α or IFN-γ treatment (Fig. 4A). These inhibitory effects of IFN-α and IFN-γ on COL1A2 mRNA expression were more prominent when tested with an activated HSC clone, CFSC-2G cells (Fig. 4A), in which COL1A2 transcription was markedly accelerated because of the activation of the TGF-β/Smad signaling pathway.[15] Western blot analyses confirmed that both IFN-α and IFN-γ treatment induced phosphorylation of Stat1 and decreased the amount of type I collagen in CFSC-2G cells (Fig. 4B).

IFN-α Antagonizes TGF-β/Smad3-Stimulated COL1A2 Transcription. CFSC-2G cells were transfected with the −17.0-15.5/350COL1A2/LUC construct containing the far-upstream enhancer sequence linked to the −350 COL1A2 promoter and treated with rat IFN-α or IFN-γ. IFN-α significantly decreased luciferase activity

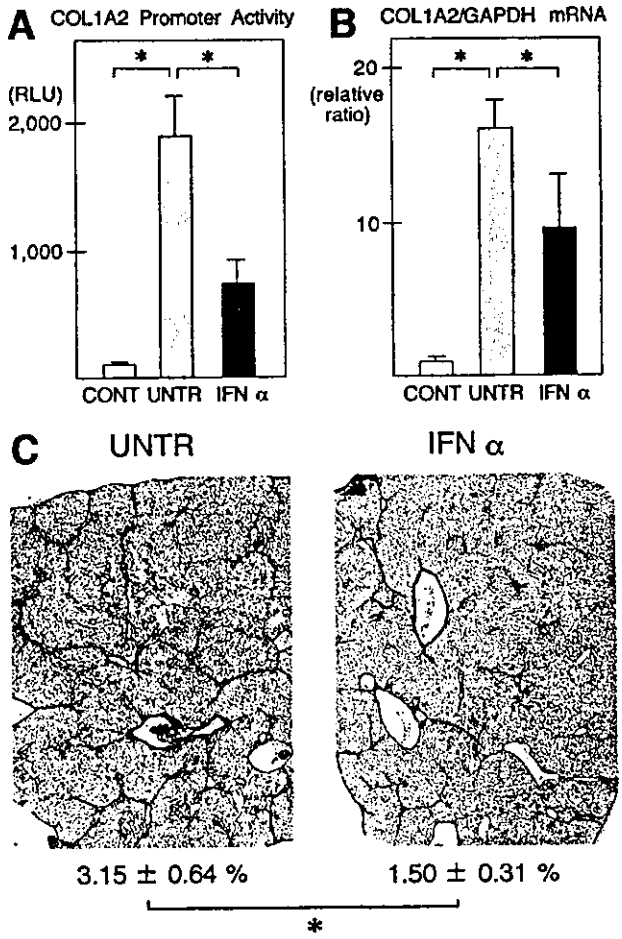

Fig. 3. Preventive effects of IFN-α treatment on the progression of carbon tetrachloride-induced hepatic fibrosis. The transgenic mice were injected intraperitoneally with 0.1 mL/kg body weight of carbon tetrachloride (CCl_4) once a week, then treated with 50,000 units/d of murine IFN-α or control saline (UNTR) 3 times a week. The mice were killed after 8 weeks of CCl_4 intoxication and subjected to (A) luciferase assays determining COL1A2 promoter activity and (B) real-time RT-PCR assays quantifying the steady-state levels of COL1A2 mRNA in liver tissue. Luciferase activity was normalized against the protein concentration of tissue homogenates, and expression levels of COL1A2 mRNA were normalized against those of GAPDH mRNA. The values are mean ± SD obtained from 5 mice in each group and expressed relative to those in the control mice without CCl_4 injection (CONT). The **asterisk** indicates that the difference between the groups is significant. (C) Sections prepared from the excised liver specimens were stained with Sirius red F3BA. Representative microscopic pictures of liver samples from untreated (UNTR) and IFN-α-treated mice are shown, together with the mean relative area of collagen deposition in liver tissue determined with the aid of computer software. The **asterisk** indicates that the difference between the groups is significant. (Original magnification ×40.)

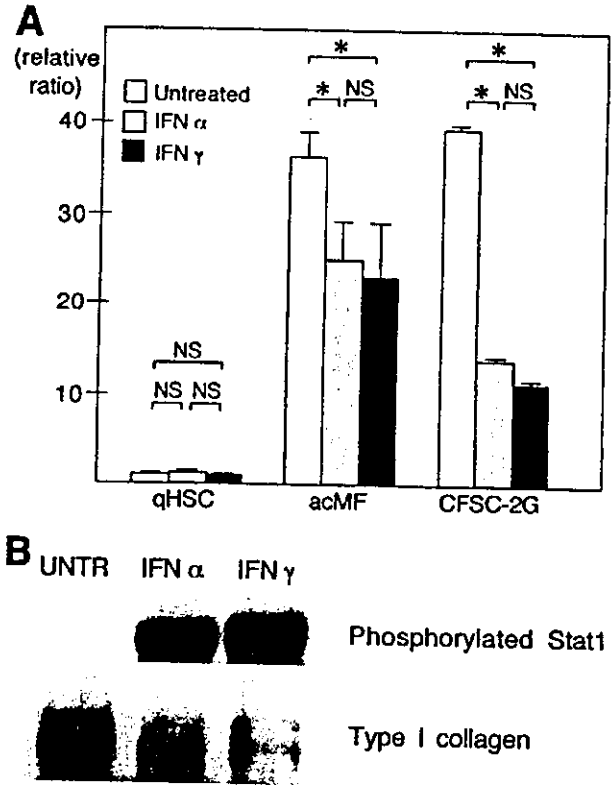

Fig. 4. Effects of IFN treatment on COL1A2 expression. (A) Quiescent hepatic stellate cells (qHSC) at day 3 after plating on plastic dishes, activated myofibroblasts (acMF) at day 7 after plating, and an activated HSC clone (CFSC-2G cells) were left untreated or treated with 500 units/mL of rat IFN-α or IFN-γ for 24 hours. The steady-state levels of COL1A2 mRNA were determined by real-time RT-PCR. Relative expression levels of COL1A2 mRNA were normalized against those of GAPDH mRNA. The values are mean ± SD obtained from 4 independent RNA preparations in each cell type and expressed relative to the activity in untreated quiescent HSC. The **asterisk** indicates that the difference between the groups is significant. (B) CFSC-2G cells were left untreated (UNTR) or treated with 500 units/mL of IFN-α or IFN-γ for 1 hour to detect phosphorylated Stat1 or for 48 hours to detect type I collagen. Whole cell lysates were prepared and immunoblotted with anti-phosphoStat1 antibodies or anticollagen type I antibodies.

driven by the COL1A2 enhancer/promoter to a similar extent as IFN-γ treatment (Fig. 5A). Administration of TGF-β into the culture media significantly increased COL1A2 transcription, and IFN-α, as well as IFN-γ, inhibited TGF-β-stimulated transcription by approximately 50% (Fig. 5A). Similar results were obtained using the −378COL1A2/LUC construct without the far-upstream enhancer sequence (Fig. 5B): we therefore used this reporter construct for further analyses. We have previously shown that Smad3 plays a key role in mediating TGF-β-elicited COL1A2 stimulation through its interaction with Sp1.[13,15,24] We therefore examined the antagonistic effects of IFN-α and IFN-γ on Smad3-stimulated COL1A2 transcription. For this purpose, CFSC-2G cells were cotrans-

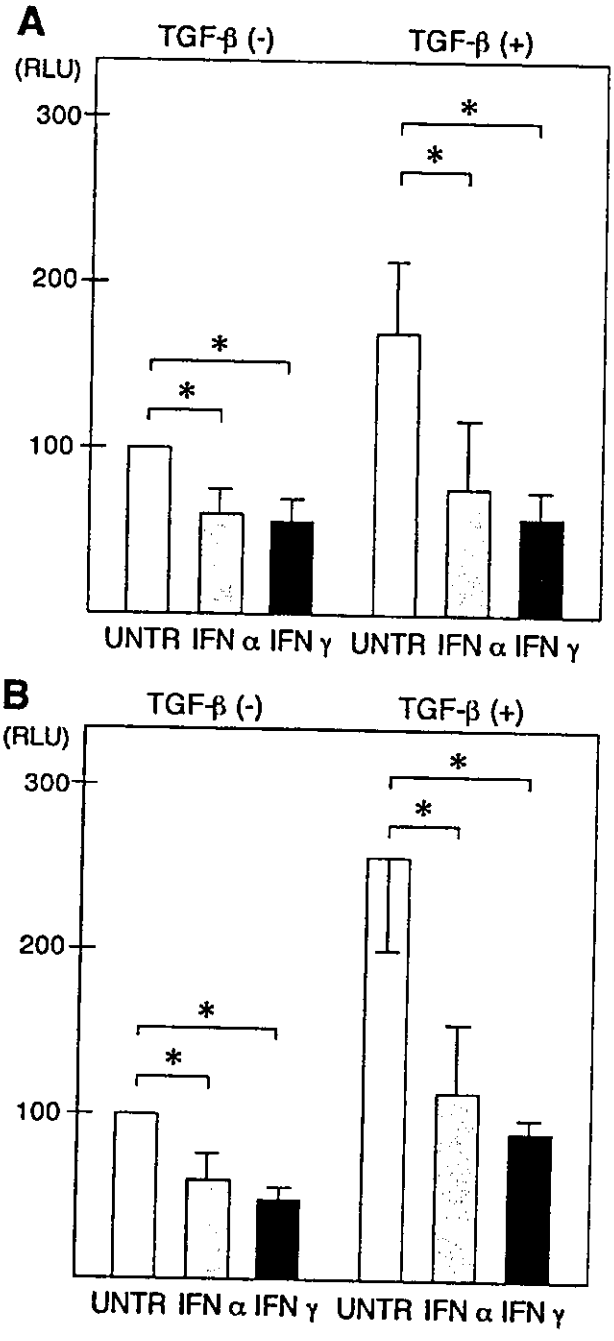

Fig. 5. Effects of IFN treatment on basal and TGF-β-stimulated COL1A2 transcription in activated hepatic stellate cells. CFSC-2G cells were transfected with the −17.0-15.5/350COL1A2/LUC (A) or the −378COL1A2/LUC construct (B) then left untreated (UNTR) or treated with 500 units/mL of rat IFN in the absence or presence of 2 ng/mL of TGF-β. The activity of each construct was normalized against the cotransfected pRLCMV. The values are mean ± SD obtained from 5 independent tests and expressed relative to the activity in untreated transfectants. The **asterisk** indicates that the difference between the groups is significant.

fected with the −378COL1A2/LUC construct and a Smad3 expression plasmid and then treated with rat IFN-α or IFN-γ. Overexpression of Smad3 significantly increased COL1A2 transcription, and both IFN-α and IFN-γ repressed this Smad3-stimulated transcription (Fig. 6A). On the other hand, IFN-α and IFN-γ did not affect COL1A2 transcription stimulated by Sp1 overexpression (data not shown). Similarly, treatment of CF37 skin fibroblasts with human IFN-α or IFN-γ significantly repressed the Smad3-stimulated COL1A2 transcription (Fig. 6B).

IFN-α Inhibits COL1A2 Transcription Through the Interaction Between Phosphorylated Stat1 and P300/CBP. It has been recently reported that TGF-β stimulates COL1A2 transcription via functional cooperation between Smad3 and p300/CBP transcriptional co-activators.[35] In addition, the competitive binding of phosphorylated Stat1 and Smad3 to a limited amount of p300/CBP has been implicated in mediating the antagonistic effects of IFN-γ on TGF-β-stimulated COL1A2 transcription.[36] Immunoprecipitation-Western blot analyses, using cell lysates prepared after transfection with a flag-tagged p300 expression plasmid, indicated that IFN-α also induced phosphorylation of Stat1 followed by its binding to p300 (Fig. 7). To further demonstrate the involvement of p300 in the inhibition of COL1A2 transcription by IFN-α, we transfected CFSC-2G and CF37 cells with an expression plasmid encoding the dominant negative form of p300. The results indicated that overexpression of the dominant negative p300 completely abolished the inhibitory effects of both IFN-α and IFN-γ on COL1A2 transcription in CFSC-2G cells (Fig. 6A) as well as in CF37 fibroblasts (Fig. 6B).

Blocking of the IFN-α Signal Represses the Inhibitory Effects on COL1A2 Transcription. In the last set of experiments, we investigated the effects of the IFN-α signal on COL1A2 transcription by utilizing expression plasmids encoding the human type I IFN receptor chains with a deletion in their intracellular domains. Because of a strict species specificity in IFN-α signaling, human CF37 cells were cotransfected with the −378COL1A2/LUC construct and the truncated IFNAR1 and/or IFNAR2 expression plasmids. Overexpression of the mutant receptors in the cells significantly increased basal COL1A2 transcription levels (Fig. 8). More importantly, suppression of COL1A2 transcription by IFN-α was not observed when the cells were transfected with the mutant plasmids (Fig. 8). These results therefore indicate that these truncated mutants act as dominant negative receptors when expressed at higher levels than the endogenous receptors and that blocking of the IFN-α signal inhibits the down-regulation of COL1A2 transcription by this cytokine.

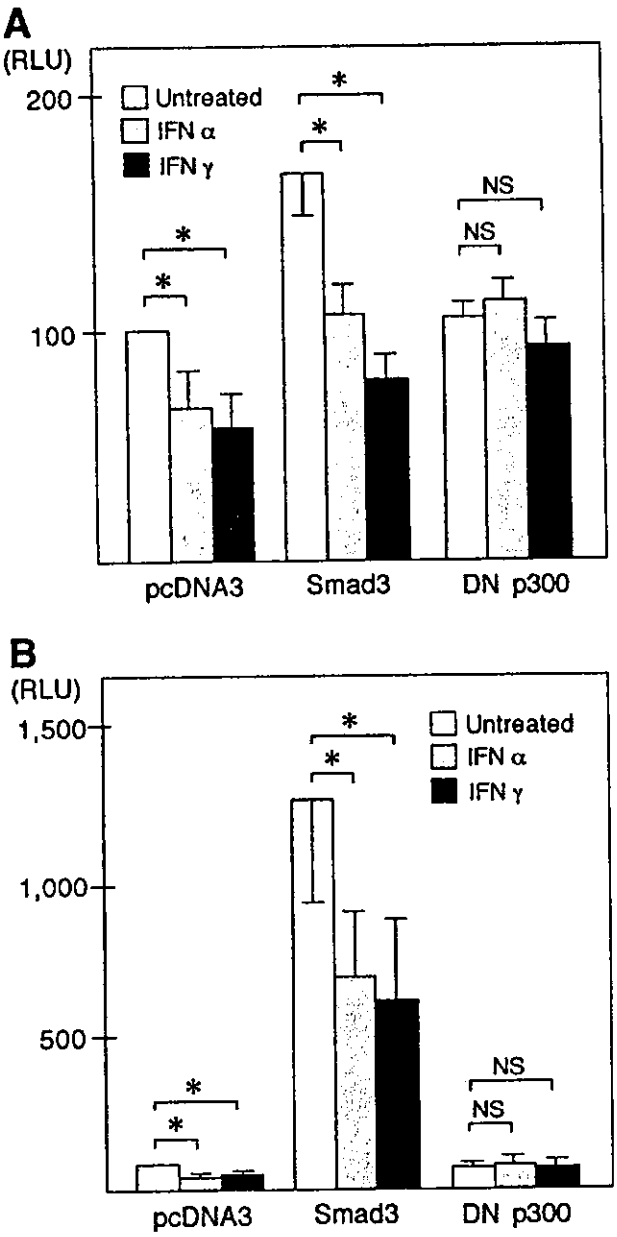

Fig. 6. Effects of the overexpression of Smad3 or the dominant negative form of p300 on COL1A2 response to IFN treatment. CFSC-2G stellate cells (A) or CF37 fibroblasts (B) were cotransfected with the −378COL1A2/LUC construct together with a control empty vector (pcDNA3) or expression plasmids encoding either Smad3 or the dominant negative form of p300 then left untreated or treated with rat (for CFSC-2G cells) or human (for CF37 cells) IFN. The values are mean ± SD obtained from 5 independent tests and expressed relative to the activity in untreated cells cotransfected with the control expression vector. The **asterisk** indicates that the difference between the groups is significant.

Discussion

Increased production of type I collagen is a common hallmark of fibrotic diseases in various organs including the liver.[1] This increase is exerted mainly by the transcrip-

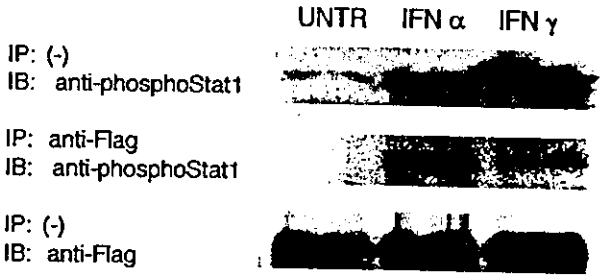

Fig. 7. Immunoprecipitation-Western blot analyses of interaction between phosphorylated Stat1 and p300. Human embryonic kidney 293 EBNA cells were transfected with a flag-tagged p300 expression plasmid. Forty-eight hours later, the cells were left untreated (UNTR) or treated with 500 units/mL of human IFNs for 1 hour. Whole cell lysates were subjected to immunoblotting (IB) with anti-phosphoStat1 antibodies either directly or after immunoprecipitation (IP) with antiflag antibodies. Whole cell lysates were immunoblotted with antiflag antibodies to confirm expression of flag-tagged p300 in transfected 293 EBNA cells.

tional up-regulation of COL1A1 and COL1A2 expression, and TGF-β is known to be a key factor in stimulating collagen gene transcription.[10] It is therefore possible that the counter-repression of TGF-β-stimulated collagen gene transcription will be a potent therapeutic means for preventing organ fibrosis by suppressing excessive collagen deposition in various tissues. In the present study, we have shown for the first time that treatment of the transgenic COL1A2 promoter/reporter mice with IFN-α, as well as IFN-γ, significantly suppressed the promoter activation induced by carbon tetrachloride injection. This inhibition of collagen promoter activation was paralleled with a decrease in the steady-state levels of COL1A2 mRNA and resulted in the suppression of hepatic fibrosis. On the other hand, there was no difference in the mean levels of serum ALT between the IFN-treated and control groups. Thus, suppression of COL1A2 promoter activation by IFN-α and IFN-γ is not due to the reduction of hepatocellular necrosis but may reflect their direct inhibitory effects on the activity of COL1A2 promoter.

It should be noted that the doses of IFN-α and IFN-γ used in the transgenic mouse experiments (50,000 units/30 g body weight) were 10 times higher, if simply compared with the pharmaceutical dose being used for humans (*e.g.*, 10,000,000 units/60-kg body weight). However, all of the previous studies working on the antifibrotic effects of IFN-α[37] and IFN-γ[38-41] in rats and mice have utilized the same or even higher doses of these cytokines. It was not clear whether IFN treatment required nonpharmaceutical dosages for suppressing hepatic fibrogenesis or if rodents were less susceptive to IFN than humans. We thus compared the suppressive effects on the promoter activation and the mean levels of serum 2,5-AS activity between the mice treated with the 2 different doses of IFN-α. The results indicated that suppression of COL1A2 promoter activation was not observed in the mice treated with the lower dose of IFN-α. In addition, the serum 2,5-AS activity was not fully elevated in this group of mice. It is therefore suggested that mice are less susceptive to IFN-α than humans and thus require higher doses of the cytokine for receiving its biologic and pharmacologic benefits.

Among the many possible mechanisms by which IFN-α suppressed collagen promoter activation *in vivo*, we focused on the inhibitory effects of IFN-α on collagen gene transcription. Cell transfection assays indicated that IFN-α was as potent as IFN-γ in suppressing basal and TGF-β–stimulated COL1A2 transcription in activated HSC, suggesting that the inhibitory actions of IFN-α and IFN-γ on COL1A2 promoter activation *in vivo* were attributed, at least in part, to their suppressive effects on COL1A2 transcription. Experiments using the dominant negative forms of the type I IFN receptor chains further confirmed the inhibitory effect of IFN-α signal on COL1A2 transcription. Overexpression of the mutant receptor increased basal COL1A2 transcription and abolished the inhibitory effects of IFN-α on gene transcription. Increased basal COL1A2 transcription after overexpressing the mutant IFN receptor may be because of a block of constitutive subthreshold expression of

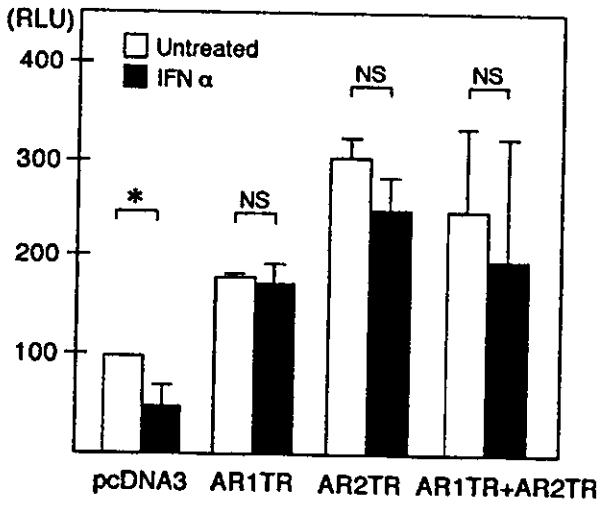

Fig. 8. Effects of the overexpression of the intracellular domain-deleted type I IFN receptor. Human skin fibroblasts CF37 were cotransfected with the −378COL1A2/LUC construct together with a control empty vector (pcDNA3) or expression plasmids encoding the intracellular domain-deleted mutants of the human type I IFN receptor chains (AR1TR and AR2TR) then left untreated or treated with human IFN-α. The values are mean ± SD obtained from 4 independent tests and expressed relative to the activity in untreated cells cotransfected with the control expression vector. The **asterisk** indicates that the difference between the groups is significant.

the wild-type receptor, which has been implicated for the rapid and full response to ligand stimulation.[42]

There have been several reports demonstrating the inhibitory effects of IFN-γ on collagen expression in *in vitro* experimental systems.[43-45] With regard to the molecular mechanisms of this inhibitory action, the IFN-γ response element was mapped to around the −160 base of the COL1A2 promoter sequence,[46] downstream of the TbRE. More recently, cross talk between the TGF-β and IFN-γ signaling pathways has been demonstrated. Specifically, IFN-γ–mediated activation of Jak1 and Stat1 leads to an induction of Smad7,[47] which inhibits the phosphorylation of Smad2 and Smad3 by the activated TGF-β receptor complex.[48] In addition, IFN-γ–activated Stat1 and its interaction with the p300/CBP coactivators has been implicated in suppressing Smad3/p300-stimulated COL1A2 transcription in skin fibroblasts.[36] In contrast to these experimental findings, little was known before this study about the mechanisms responsible for the inhibitory effects of IFN-α on collagen expression, except that it inhibits proliferation of HSC[45] and that IFN-α therapy reduces the concentration of TGF-β in both plasma and liver tissue.[9]

Binding of IFN-α to the type I IFN receptor complex, composed of IFNAR1 and IFNAR2, initiates the cascade of signal transduction events. The ligand-bound type I IFN receptor complex activates the Jak1 and Tyk2 tyrosine kinases, which in turn phosphorylate Stat1 and Stat2 and lead to the formation of the Stat1/Stat2/p48 complex.[49] On the other hand, IFN-γ binds to a distinct type II IFN receptor complex, composed of IFNGR1 and IFNGR2. The IFN-γ–bound type II IFN receptor complex activates the Jak1 and Jak2 kinases, resulting in phosphorylation and dimer formation of Stat1.[49] It was thus originally considered that IFN-α and IFN-γ had independent signaling pathways from each other. However, since IFN-α also induces the formation of Stat1 dimers to some extent in addition to the Stat1/Stat2/p48 complex, some of the actions of IFN-α could be exerted through the same mechanisms as IFN-γ.[50] In addition, it has been recently reported that IFN-α/β signaling contributes to IFN-γ signaling through the association of IFNAR1 with IFNGR2,[42] indicating a cross talk between the type I and type II IFN receptor signals. The results of the present study have clearly indicated that IFN-α, as well as IFN-γ, induces phosphorylation of Stat1 followed by its binding to p300 and that the inhibitory effects of both IFN-α and IFN-γ on COL1A2 transcription are p300 dependent. From these findings, it could be argued that the competitive binding of Stat1 and Smad3 to p300 is a common mechanism exerting the inhibitory actions of IFN-α and IFN-γ on COL1A2 transcription.

In summary, the present study is the first to demonstrate that IFN-α and its intracellular signal downregulate COL1A2 transcription *in vitro* and suppress activation of the COL1A2 promoter induced by carbon tetrachloride administration *in vivo*. These results not only lead to better understanding of the mechanisms controlling collagen expression by growth factors and cytokines but also provide a molecular basis for antifibrotic effects of IFN-α that is widely used for the treatment of chronic viral hepatitis.

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c-JUN NH_2-TERMINAL KINASE PATHWAY IS INVOLVED IN CONSTITUTIVE MATRIX METALLOPROTEINASE-1 EXPRESSION IN A HEPATOCELLULAR CARCINOMA-DERIVED CELL LINE

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Transcription factor c-Jun serves for cellular proliferation, survival, differentiation and transformation and is recognized as an important factor in cancer development, including hepatocellular carcinoma (HCC). The purpose of present study is to determine the involvement of c-Jun in matrix metalloproteinase-1 (MMP-1) expression, which is previously reported by us to be expressed only in the early stage of human HCC showing stromal invasion. Of 5 human HCC cell lines examined, only HLE cells revealed mRNA and protein expression as well as enzymatic activity of MMP-1. Transient transfection of an MMP-1 promoter/luciferase construct (including 4.4 kb full promoter region) into HLE and HCC-T cells (MMP-1 nonproducer) showed that high promoter activity was observed only in HLE cells without inducers, and that this promoter activity was still observed when a shorter 0.6 kb proximal promoter construct was transfected. The 0.6 kb promoter region contained 3 AP-1 sites, and c-jun mRNA was constitutively expressed in HLE cells without inducers. Furthermore, phosphorylated c-Jun and c-Jun NH_2-terminal kinase (JNK) were detected in HLE cells. Promoter activity of the 0.6 kb construct was suppressed with SP600125, a potent inhibitor of JNK, but not with PD98059 and SB203580, potent inhibitors of MEK1/2 and p38, respectively. The inhibitory effect of SP600125 was also observed at protein expression level and in enzymatic activity of MMP-1. Taken together, this study suggests that the JNK pathway is involved in the expression of MMP-1 in HCC cells and may represent a new functional role of c-Jun for HCC development.

Key words: *hepatocellular carcinoma; matrix metalloproteinase-1; c-Jun; MAP kinase inhibitors; JNK pathway*

Invasion and metastasis are the major causes of treatment failure in patients with cancer. Enzymatic degradation of different macromolecular components of the extracellular matrix (ECM), which is an essential step in the process of invasion and metastasis, plays a key role in the dissemination of cancer cells.[1,2] Of several proteolytic enzymes, it has been clarified that metalloproteinases (MMPs) are responsible for ECM destruction, and that they participate in cancer cell invasion and metastasis.[3,4]

We previously showed that only well-differentiated cancer cells of early hepatocellular carcinoma (HCC), smaller than 2 cm in diameter, express matrix metalloproteinase-1 (MMP-1) by *in situ* hybridization and immunohistochemistry.[5] Early HCC is usually described as well-differentiated carcinoma[6] and stromal invasion of cancer cells is a common finding in early HCC.[7] As HCC is usually associated with liver fibrosis/cirrhosis,[8–10] where type 1 collagen is mainly deposited, it is quite likely that early HCC cells invade surrounding fibrous tissue by secreting MMP-1. MMP-1 expression is only detected in the early stage of HCC, which is coincident with clinical feature of HCC. In advanced stage of HCC, they are usually encapsulated with fibrous tissue and invade surrounding fibrous tissues no longer.

A recent study revealed that MMP-2 and membrane type 1-matrix metalloproteinase (MT1-MMP) can cause the cells to proliferate[11] besides their well-known original function, that is, ECM resolution. Moreover, MT1-MMP was reported to increase cell mobility.[12] MMP-1 was also supposed to cause the proliferation of hepatocytes in the rat fibrotic liver infected with recombinant adenovirus harboring human MMP-1 gene.[13]

c-Jun and activated c-Jun by a phosphorylation cascade of mitogen-activated protein kinase (MAPK) families have been shown to play an important role in embryonic cell differentiation, apoptosis and proliferation as well as carcinogenesis of hepatocytes.[14] The phosphorylation of c-Jun is conducted by c-Jun NH_2-terminal kinases (JNK) among 4 distinctly regulated groups of MAPK pathways; the other 3 groups are extracellular signal-related kinases (ERK)-1/2, p38 proteins and ERK5.[15–18]

Several recent studies describing involvement of c-Jun in the early stage of HCC development[14] or in liver regeneration after partial hepatectomy[19] remind us that JNK pathway has a key role in the MMP-1 gene expression in HCC. MMP-1 gene expression is truly known to be regulated by c-Jun, but the regulation of MMP-1 gene expression by JNK pathway in HCC cells remains unknown. The present study has shown for the first time that one HCC cell line, which constitutively expresses MMP-1 without any stimulators such as phorbor ester, is under control of transcriptional regulation of the MMP-1 gene transcription via the activation of c-Jun through JNK pathway. These results may indicate an additional functional role of c-Jun in HCC development.

MATERIAL AND METHODS

HCC cell lines

Human HCC cell lines HLE,[20] PLC/PRF/5[21] and Huh-7[22] were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). HCC-M and HCC-T were previously established by us.[23,24] HCC-M, HLE, PLC/PRF/5 and Huh-7 were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), nonessential amino acids and antibiotics. HCC-T was cultured with RPMI-1640 containing 10% FBS and antibiotics.

Reagents

c-Jun NH_2-terminal kinase inhibitor SP600125 was purchased from Calbiochem-Novabiochem (San Diego, CA). MEK/ERK inhibitor PD98059 and p38 inhibitor SB203580 were obtained from Sigma Chemical (St. Louis, MO).

RNA isolation and RT-PCR

Isolation of total RNA and RT-PCR were performed as described previously.[25] To amplify each gene, a pair of sense and antisense primers (Table I) were chosen with the help of a computer program (Oligo 5.0, Primer analysis software, National Bio-

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TABLE I – PRIMERS FOR RT-PCR AMPLIFICATION

Gene		Nucleotide sequence	Annealing temperature (°C)	Cycle	Product (bp)
Albumin	Sense	5'-CCCCGGAACTCCTTTTCTTTG-3'	56	40	675
	Antisense	5'-CATCGAACACTTTGGCATAGCA-3'			
HNF-4	Sense	5-'CTGCTCGGAGCCACAAAGAGATCCATG-3'	57	30	371
	Antisense	5-'ATCATCTGCCACGTGATGCTCTGCA-3'			
AFP	Sense	5'-CGCTGGAACGTGGTCAATGTA-3'	56	40	706
	Antisense	5-'CACCCTGAGCTTGGCACAGA-3'			
MMP-1	Sense	5'-GGTGCCCAGTGGTTGAAAAAT-3'	57	35	716
	Antisense	5'-CATCACTTCTCCCCGAATCGT-3'			
MMP-2	Sense	5'-TCTTCCCTCGCAAGCCCAAGT-3'	57	35	685
	Antisense	5'-ACAGTGGACATGGCGGTCTCAG-3'			
MMP-9	Sense	5'-TGGGCTACGTGACCTATGAC-3'	59	35	200
	Antisense	5'-CAAAGGTGAGAAGAGAGGGC-3'			
TIMP-1	Sense	5'-TTCTGCAATTCCGACCTCGTC-3'	58	30	385
	Antisense	5'-GCAGTTTGCAGGGGATGGATA-3'			
c-jun	Sense	5'-CCTGTTGCGGCCCCGAAACT-3'	62	30	495
	Antisense	5'-ACCATGCCTGCCCCGTTGAC-3'			
c-fos	Sense	5'-TTTGCCTAACCGCCACGATGAT-3'	62	30	500
	Antisense	5'-TTGCCGCTTTCTGCCACCTC-3'			
G3PDH	Sense	5'-ACCACAGTCCATGCCATCAC-3'	62	23	452
	Antisense	5'-TCCACCACCCTGTTGCTGTA-3'			

science, Plymouth, MN). Each target gene was amplified in the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). The initial denaturation was at 94°C for 2 min, followed by each cycle of reaction at 94°C for 30 sec, at the described annealing temperature for 30 sec (Table I), and at 72°C for 30 sec, and followed by postextension at 72°C for 7 min. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which was used as an internal control, was amplified for 23 cycles as previously described.[25] The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

Zymography

To examine the gelatinolytic activity, gelatin zymography was performed as described before[25] with a slight modification. After cells were cultured in serum-free media for 48 hr, aliquots of the culture media from HCC cells were mixed with 4 × gel loading buffer (10% SDS, 4% sucrose, 0.25 M Tris-HCl, pH 6.8, 0.1% bromphenol blue) without boiling, then electrophoresed on a 10% polyacrylamide gel containing 1 mg/mL of gelatin. The volume of media loaded was adjusted according to the cell number. Gels were scanned in a digital scanner and densitometric measurement was performed with NIH Image software (version 1.55).

Nucleotide sequence analysis for single nucleotide polymorphism at −1607 bp

To detect the known 1G/2G polymorphism at −1607 bp in the MMP-1 promoter region, PCR amplification was performed with a pair of primers, M-F (5'-ACATGTTATGCCACTTAGAT-3'; −1654/−1635) and M-R (5'-TCCCCTTATATGGATTCCTGTT-3'; −1536/−1517), followed by nucleotide sequence analysis.[26]

Plasmid constructs

A fragment encompassing the essential sequence for transcriptional activity of MMP-1 promoter was amplified from genomic DNA isolated from HCC-T cells using the following primers: sense, 5'-TTTCAAATCCATCTCAAATTCACA-3' (−4363/−4340); antisense, 5'-ACTGGCCTTTGTCTTCTTTCTCAG-3' (+49/+72). *Bgl*II digestion of the resulting 4,429 bp PCR product (−4363/+72; 4.4 kb construct) yielded a 1.2 kb fragment (−1196/+72), which was cloned into the pGL3 Basic vector (Promega, Tokyo, Japan) that had been treated to have the same termini. A 5' deletion promoter 0.6 kb construct (−522/+72) was generated from this construct (−1196/+72) by using convenient restriction site (*Kpn*I at −517 bp). Those chimeric constructs were sequenced and found to be identical to the previously reported sequence[27] except that our sequence from HCC-T had a "T" at site −320 nucleotide, where the published sequence contained a "C" at that site. Expression plasmid pCMV-jun was the kind gift of Dr. Tom Curran.

Transient transfection and assessment of promoter activity

The MMP-1 promoter/luciferase constructs were transfected into HLE and HCC-T cells using the calcium phosphate-DNA coprecipitation method. In some experiments, human MMP-1 promoter/luciferase reporter gene construct (−522/+72; 0.6 kb) was cotransfected with pCMV-jun expression vector into HCC-T cells. Total amount of tranfected DNA was adjusted with pCMV empty vector. Transfection efficiency was normalized by using pRL-CMV vector (Promega) as an internal control. Five hours after transfection, the cells were treated with 15% glycerol for 105 sec, then incubated for 48 hr. Firefly and Renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega). Cell transfections and luciferase assays were repeated independently more than 3 times, each performed in duplicate.

Western blot analysis for MMP-1 and phosphorylated c-Jun

For the detection of MMP-1, culture media described above were also used for Western blot analysis. The concentration of protein was measured with DC protein assay Kit (Bio-Rad, Richmond, CA). Samples were mixed with 4 × SDS sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 20% glycerol, 5% β-mercaptoethanol) and equal amount of protein per lane was run on a 10% SDS-PAGE and transferred onto a PVDF membrane (Amersham Biosciences, Buckinghamshire, U.K.). Blots were incubated with anti-MMP-1 antibody (Daiichi Fine Chemical, Takaoka, Japan) at a dilution of 1:1,000 for 2 hr, followed by incubation with rabbit antimouse IgG second antibody (Dako, Glostrup, Denmark) at a dilution of 1:2,000 for 1 hr at room temperature. The proteins were visualized by chemiluminescence by using ECL Plus detection kit (Amersham Biosciences) according to the manufacturer's instruction. For the detection of phosphorylated c-Jun, PhosphoPlus c-Jun (Ser63) II and c-Jun (Ser73) Antibody Kit (Cell Signaling Technology, Beverly, MA) was used according to the manufacturer's instruction. For the confirmation of equal loading, blots were reprobed with anti-β-actin antibody (Sigma Chemical).

In vitro *kinase assay*

Phosphorylated JNK and total-JNK were detected with Fast Activated Cell-Based ELISA (FACE) JNK Kit (Active Motif North America, Carlsbag, CA) following manufacturer's instructions. Briefly, about 1,000 cells per well were seeded in 2 96-well plates as replicates and incubated for 48 hr and the cells were fixed. One plate was treated with the antiphospho-JNK antibody, while the other plate was treated with anti-JNK antibody. The relative

number of cells in each well was then determined through use of the Crystal Violet reagent. Once the phospho-JNK and total JNK signals were normalized for cell number, a comparison of the ratio of phosphorylated JNK to total JNK for each of the cell growth conditions was determined.

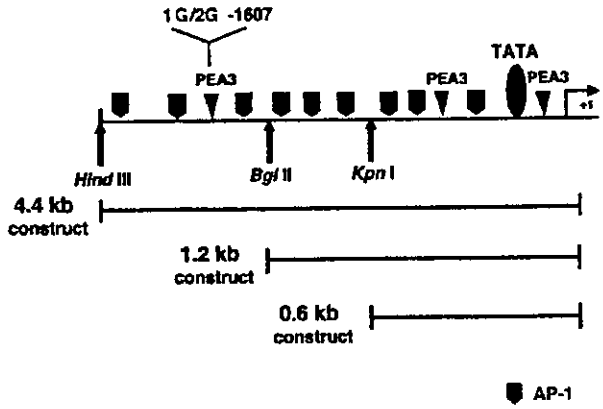


FIGURE 2 – Schematic representation of human MMP-1 promoter constructs. Three convenient restriction sites, *Hind*III, *Bgl*II and *Kpn*I, were used to generate the 4.4, 1.2 and 0.6 kb promoter constructs, respectively, which were transfected into HLE and HCC-T cells to determine the promoter activity. Three AP-1 sites, 2 PEA-3 sites and TATA box are included in the 0.6 kb promoter construct. The "Y" indicates functional polymorphism in MMP-1 promoter. The bent arrow represents the beginning of transcription. Modified from Benbow and Brinckerhoff.[18]

Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analysis was performed using Mann-Whitney test. *p*-values less than 0.05 were considered statistically significant.

RESULTS

MMP-1 expression and cell marker characteristics in HCC cell lines

Among the HCC cell lines examined, definite expression of MMP-1, MMP-2 and MMP-9 mRNAs was found only in HLE cells, while these 3 MMPs were not detected in HCC-M and Huh-7 cells (Fig. 1*a*). HCC-T and PLC/PRF/5 cells showed a slight positive transcription of MMP-2. TIMP-1 mRNA was expressed in all cell lines in the present study (Fig. 1*a*). Gelatin zymography revealed definite bands at 76, 68 and 53 kDa only in HLE cells, which corresponded to enzymatic activity of MMP-9, MMP-2 and MMP-1, respectively (Fig. 1*b*). The other 4 cell lines did not show any bands.

Five cell lines were established from patients with different differentiation stages of characteristics. Both PLC/PRF/5 and Huh-7 cells showed positive mRNAs of both albumin and HNF-4,

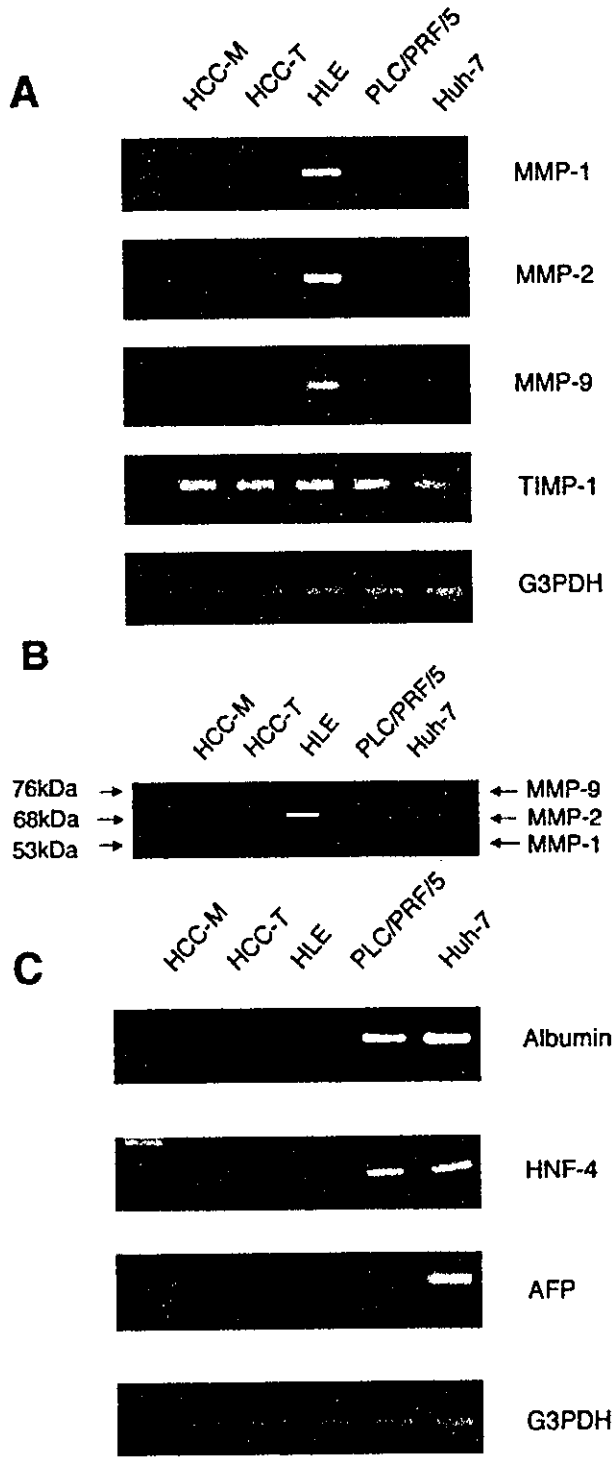


FIGURE 1 – MMPs and TIMP-1 expression in HCC cell lines. (*a*) MMP-1, MMP-2, MMP-9 and TIMP-1 expressions in 5 HCC cell lines were detected by RT-PCR analysis. Total RNA was extracted from HCC cells and 1 μg of total RNA was used as a template as described in text. The strong bands of MMP-1, MMP-2 and MMP-9 were observed only in HLE cell line. HCC-T and PLC/PRF/5 cells showed a slight positive transcription of MMP-2. The expression of TIMP-1 was seen in every cell line. G3PDH (bottom) was used as an internal control. (*b*) Gelatin zymography of conditioned media from cultured 5 HCC cell lines. Semiconfluent HCC cells grown in 60 mm tissue culture plates were replaced with serum-free media and cells were cultured for a further 48 hr. Bands of negative staining indicated zones of enzyme activities. Gelatinolytic activity of MMP-1, MMP-2 and MMP-9, which corresponded to 53, 68 and 76 kDa bands, respectively, were seen only in HLE cells. (*c*) Cell marker characteristics in HCC cell lines. The expressions of albumin, HNF-4 and α-fetoprotein (AFP) were also assayed by RT-PCR analysis. The distinct albumin and HNF-4 mRNA was observed in PLC/PRF/5 and Huh-7 cells. The strong band of AFP was found in Huh-7 cells.

TABLE II – RELATIONSHIONSHIP BETWEEN POLYMORPHISM IN MMP-1 PROMOTER AND EXPRESSION

Genotype	HCC-M (2G/2G)	HCC-T (2G/2G)	HLE (1G/2G)	PLC/PRF/5 (1G/2G)	Huh-7 (2G/2G)
MMP-1 expression by RT-PCR	–	–	+	–	–
Enzymatic activity of MMP-1 by zymography	–	–	+	–	–

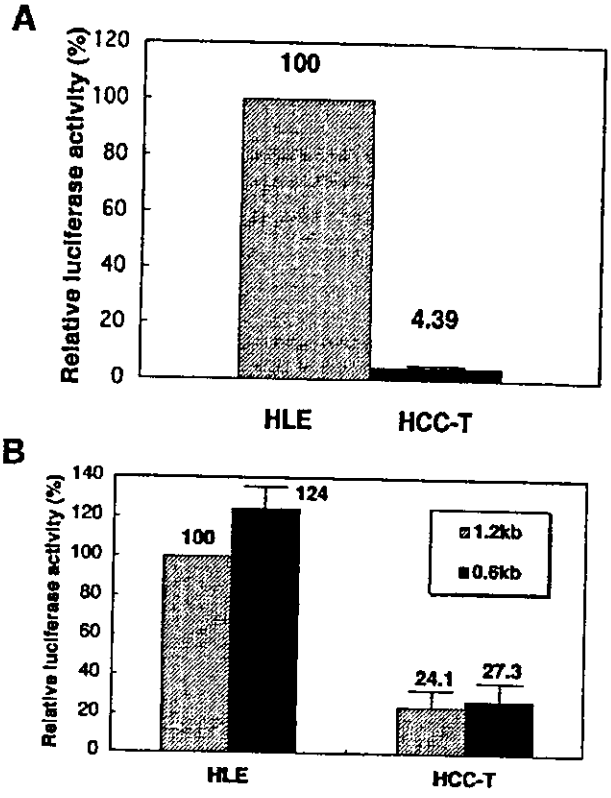


FIGURE 3 – Basal transcriptional activity of MMP-1 promoter in HLE and HCC-T cells. As described in text, HLE and HCC-T cell lines were transfected with human MMP-1 promoter/luciferase reporter gene construct (4.4 kb; *a*) and 2 5' deletion constructs (1.2 or 0.6 kb; *b*) together with pRL-CMV. After transfection, cells were incubated for 48 hr. Relative luciferase activities (mean ± SD) were normalized for pRL-CMV activity and calculated as percent of the HLE promoter activity, which was transfected with 1.2 kb construct. Transfections and assays were performed independently 4 to 6 times, each run in duplicate.

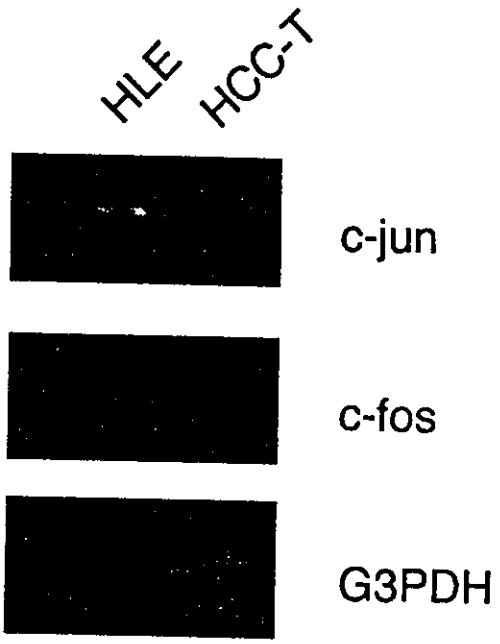


FIGURE 4 – c-jun and c-fos expression in HLE and HCC-T cells. c-jun and c-fos expression was detected with RT-PCR analysis. HLE cells, but not HCC-T cells, expressed c-jun mRNA without exogenous stimulation. c-fos mRNA was not observed in either HLE or HCC-T cells.

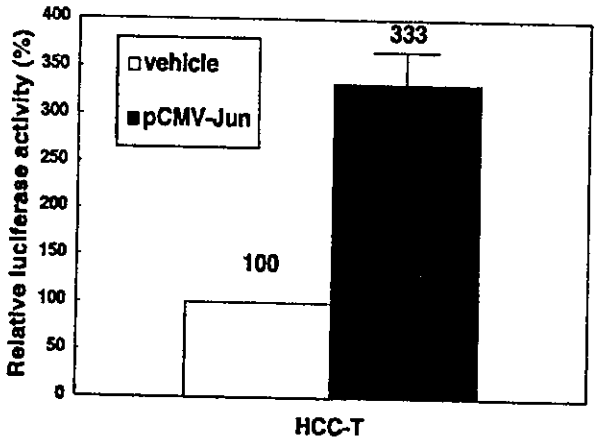


FIGURE 5 – Effect of c-Jun overexpression on HCC-T promoter activity. Human MMP-1 promoter/luciferase reporter gene construct (0.6 kb; 2.5 μg) was cotransfected with pCMV-jun vector (2.5 μg) in HCC-T cells and the effect of c-Jun on the activation of MMP-1 transcription was examined. Total amount of transfected DNA was adjusted with pCMV (vehicle) to 7.5 μg. After transfection, cells were incubated for 48 hr. Relative luciferase activities (mean ± SD) were shown after normalization for pRL-CMV activity. Transfections and assays were performed independently 4 times, each run in duplicate.

while HCC-M, HCC-T and HLE cells did not show these mRNAs (Fig. 1*c*). These results are coincident with the original characteristics of those cell lines, indicating PLC/PRF/5 and Huh-7 cells as well-differentiated HCC cells and HCC-M, HCC-T and HLE cells as less differentiated HCC cells.

Analysis of −1607 nucleotide polymorphism in MMP-1 promoter region

A single nucleotide polymorphism at −1607 nucleotide in the MMP-1 promoter region creates a PEA-3-binding site (5'-GGAA-3') as shown in Figure 2 and affects the transcriptional level of MMP-1.[28] HCC-M, HCC-T and Huh-7 cells possessed 2G/2G genotype, while HLE cells showed 1G/2G genotype (Table II). There was no correlation between the genotypes and MMP-1 expression levels, indicating that another mechanism may regulate gene expression of MMP-1 in HCC cells.

Transcriptional activity of MMP-1 promoter in HLE and HCC-T cells

In order to clarify the contribution of transcriptional factors in HLE cells, 4.4 kb MMP-1 promoter construct, which covered the

entire promoter region, was transfected into HLE and HCC-T cells. The MMP-1 promoter activity of HLE cells was much higher than that of HCC-T cells (Fig. 3*a*). These data indicate that some transcriptional factors were involved in constitutive MMP-1 expression in HLE cells. Then, to determine the region responsible for the difference in the promoter activity between HLE and HCC-T cells, 2 5′ deletion constructs (1.2 or 0.6 kb) were prepared and transfected into HLE and HCC-T cells. The promoter activity in HLE cells was higher than that in HCC-T cells (about 4- or 5-fold) not only when transfected with the 1.2 kb construct but also with the minimal 0.6 kb construct (Fig. 3*b*). Since 3 AP-1 sites were present within the 0.6 kb promoter construct (Fig. 2), transcription factor AP-1 may be responsible for MMP-1 expression in HLE cells.

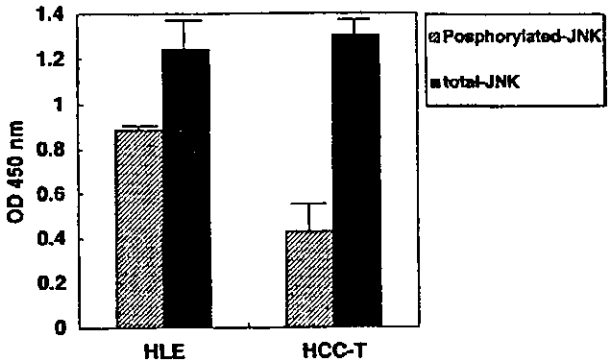


FIGURE 6 – Measurement of phosphorylated and total JNK. HLE and HCC-T cells were cultured in 96-well plates for 48 hr and the cells were fixed. Total and phospho-JNK were each assayed in triplicate using antiphosphorylated and anti-JNK antibodies from the FACE JNK Kit. Data were plotted (mean ± SD) after correction for cell number (performed through use of Crystal Violet). Note that the level of total JNK was almost the same with HLE and HCC-T cells.

Different c-jun and c-fos expression in HCC cells

Next we examined the gene expression of c-jun and c-fos in HLE and HCC-T cells to examine the contribution of AP-1 protein to MMP-1 expression in HLE cells. The definite band of c-jun was seen in HLE cells, while no band was detected in HCC-T cells (Fig. 4). c-fos gene expression was not noted in these cell lines. In order to determine the contributions of c-Jun to MMP-1 promoter activity, we transfected c-Jun expression vector (pCMV-jun vector) into HCC-T cells and measured the MMP-1 promoter activity. When c-Jun was overexpressed in HCC-T cells, 3.3-fold transcriptional activation of MMP-1 promoter was detected (Fig. 5).

JNK activity and c-Jun phosphorylation in HCC cells

To confirm the constitutive activation of JNK in HLE cells, phosphorylated and total JNK were measured with the FACE Kit. The amount of phosphorylated JNK protein in HLE cells was more than twice as much as that in HCC-T cells (Fig. 6), though the level of total JNK was almost the same in the 2 cell lines.

Then, phosphorylated c-Jun was analyzed by Western blot analysis. Phosphorylated c-Jun was detected with both phospho-c-Jun antibody (Ser63) and phospho-c-Jun antibody (Ser73) only in HLE

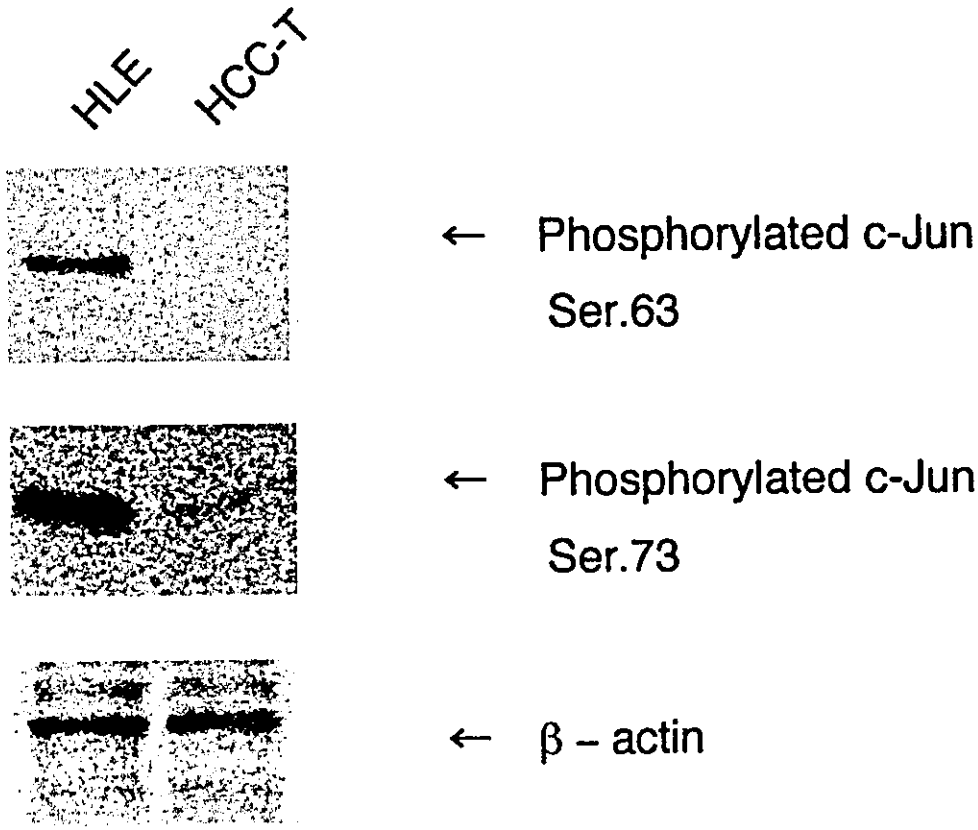


FIGURE 7 – Western blot analysis for phosphorylated c-Jun. Proteins were extracted from HLE and HCC-T cells and assessed by Western blot analysis using 2 antibodies to phosphorylated c-Jun (Ser63 and Ser73). In HLE cells, c-Jun was phosphorylated at Ser63 and Ser73. Blots were reprobed for β-actin to confirm the equal protein loading.

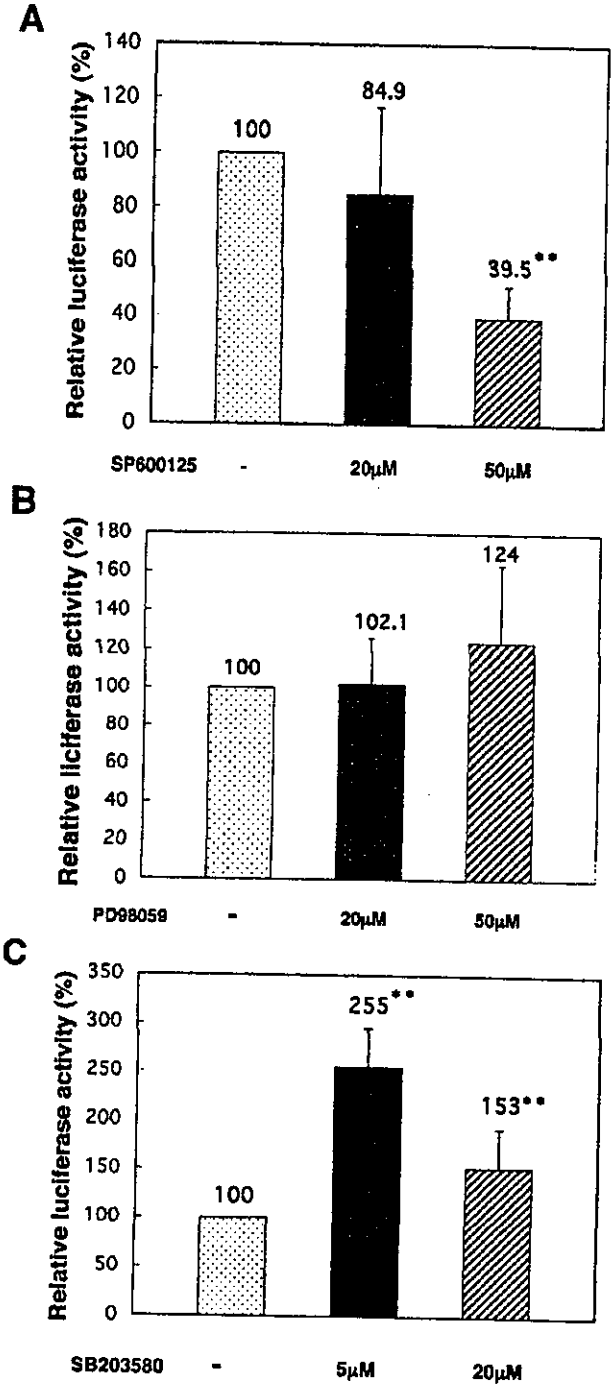
cells, but not in HCC-T cells (Fig. 7). These data suggested that the JNK pathway is constitutively activated in HLE cells, resulting in the high MMP-1 expression in the cells.

Effects of MAP kinase inhibitors on MMP-1 promoter activity

HLE cells had the ability to produce MMP-1 without exogenous stimuli and expressed c-jun (Fig. 4). As c-Jun activation is achieved by MAP kinase families, we used 3 MAP kinase inhibitors to determine the responsible enzyme of MAP kinase families for constitutive MMP-1 gene expression in HLE cell line. It was clearly noted that JNK inhibitor SP600125 (50 μM) reduced the promoter activity of 0.6 kb construct to approximately 40% (Fig. 8*a*), while either MEK/ERK inhibitor PD98059 (20 and 50 μM) or p38 inhibitor SB203580 (5 μM and 20 μM) did not reduce the promoter activity of MMP-1 (Fig. 8*b* and *c*), further indicating that c-Jun activation through JNK pathway may participate in constitutive MMP-1 expression in HLE cells.

Effect of JNK inhibitor on MMP-1 protein expression and gelatinolytic activity

To examine the inhibitory effect of SP600125 on MMP-1 expression directly, we performed Western blot analysis and zymography after treatment of HLE cells with SP600125. Both MMP-1 protein expression and gelatinolytic activity were actually reduced 48 hr after the addition of SP600125 (Fig. 9). In contract, the effect of SP600125 on MMP-2 expression was less effective. This result may reflect the number of AP-1-binding sites among the promoter regions of MMP-1 and MMP-2.



DISCUSSION

Clarification of the regulatory mechanism of MMP-1 gene expression in HCC cells is quite important because MMP-1 expression was observed only in an early stage of HCC[5] and this phenomenon is accountable to the histologic feature suggesting stromal invasion at the early stage of HCC. In advanced stage of HCC, tumor is encapsulated and does not express MMP-1. In general, HCC tumor cells show very slight atypia in the early stage and they are sometimes indistinguishable from normal hepatocytes. As the tumor grows, dedifferentiation occurs in HCC cells, where well-differentiated cancer cells are replaced by less differentiated cancer cells.[6,29] Since HCC usually arises from a rigid cirrhotic liver in which interstitial collagen is deposited predominantly,[8,10,30] tumor cells have to dissolve surrounding fibrous tissue for their expansion by producing MMP-1.

First we examined the expression of MMP-1 in cultured human HCC cell lines, which were derived from various stages of differentiation, and found that MMP-1 gene and protein expressions as well as enzymatic activity were observed only in HLE cells without any stimulation. It is true that HLE cells do not produce albumin or α-fetoprotein, but they may lie in a process of dedifferentiation of HCC cells and be considered as special cells to maintain early property of HCC cells. In any event, investigation of the mechanism of MMP-1 gene expression in HLE cells should provide precious clue to clarify the regulatory mechanism of MMP-1 expression in HCC. We thus used HLE cells and nonproducing HCC-T cells as a control for further analysis to explore the regulatory mechanisms of MMP-1 expression.

Transient transfection assay with the 0.6 kb construct (−522/+72) showed about 5-fold higher activity in HLE cells than that in HCC-T cells. Within this promoter region, there are 3 AP-1 sites at −436, −181 and −72 bp (Genbank accession number AF023338), 1 PEA-3 site (polyomavirus enhancer A-binding protein-3), 1 reversed PEA-3 site, CACCC box, TTCA motif and

FIGURE 8 – Effect of MAP kinase inhibitors on MMP-1 promoter activity. Inhibition of MMP-1 promoter activities by 3 MAP kinase inhibitors was investigated in constitutively MMP-1-expressing HLE cells. Treatment of HLE cells with SP600125 (*a*) showed a significantly decreased activity in a dose-dependent fashion, but treatment with PD98059 (*b*) or SB203580 (*c*) did not reduce the MMP-1 promoter activity. Details were described in text. Before transfection, HLE cells were cultured for 24 hr in the presence of SP600125 (20 and 50 μM; JNK inhibitor), PD98059 (20 and 50 μM; MEK/ERK inhibitor), or SB203580 (5 and 20 μM; p38 inhibitor). After transfection with human MMP-1 promoter/luciferase reporter gene construct (0.6 kb) together with pRL-CMV, HLE cells were incubated for 48 hr in the presence of the same concentrations of each inhibitor. Transfections and assays were performed independently 4 to 6 times, each run in duplicate. Statistical significance was defined as $p < 0.01$ (double asterisk).

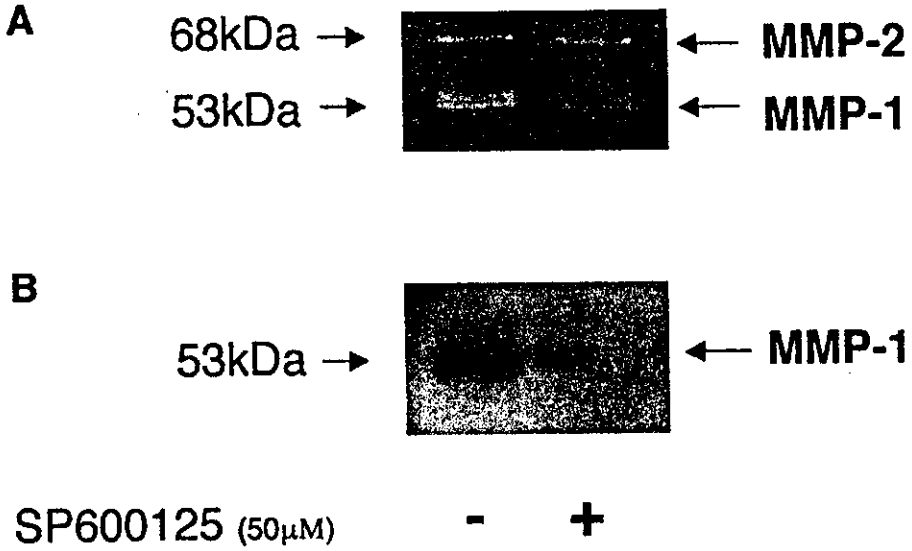


FIGURE 9 – Effect of JNK inhibitor on MMP-1 expression and gelatinolytic activity in HLE cells. HLE cells were cultured in serum-free media for 48 hr in the presence of 50 μM JNK inhibitor SP600125 and its effects on the expression of MMP-1 was analyzed by gelatin zymography (*a*) and Western blot analysis (*b*). Representative films are shown from 3 independent experiments. The enzymatic activity and protein level of MMP-1 were reduced to 62.7% ± 9.8% ($p < 0.05$) and 37.4% ± 5.8% ($p < 0.01$), respectively. The enzymatic activity of MMP-2 was slightly reduced to 92% ± 8.2%, but the statistical significance was not detected.

TATA box.[27] We could not detect any mutations in these transcription factor-binding elements in HCC cell line. Moreover, RT-PCR analysis showed that the expression of c-jun, but not c-fos, was detected without exogenous stimulation in HLE cells. Therefore, we hypothesized that in HLE cells, MMP-1 transcription was constitutively activated through the c-Jun-binding to the AP-1-binding sites. The AP-1 transcription factor itself can be formed by either the dimerization of Jun family members or the formation of Jun/Fos heterodimers.[31–33] Increased MMP-1 promoter activity observed in HCC-T cells tranfected with c-jun expression vector indicates that only c-Jun is enough for MMP-1 expression via AP-1-binding sites.

JNKs phosphorylate specific sites (ser63 and ser73) of c-Jun and enhance the transcriptional activity of AP-1, whose phosphorylation is induced after exposure to ultraviolet irradiation, growth factors, or cytokines.[34,35] To confirm activation of the JNK pathway in HLE cells, we next examined phosphorylation of JNK and c-Jun with Western blot analysis. Although we could not find any difference in the amount of total JNK between HLE and HCC-T cells, phosphorylated JNK in HLE cells was twice as much as that in HCC-T. Moreover, a strong band of phosphorylated c-Jun was detected in HLE cells, while no band or very faint band, if any, was found in HCC-T cells.

Transient transfection assays with 3 independent MAP kinase inhibitors[36,37] revealed that JNK- specific inhibitor SP600125 (50 μM) reduced the promoter activity of MMP-1 to approximately 40% in HLE cell line, while neither MEK/ERK-specific inhibitor PD98059 (20 and 50 μM) nor p38-specific inhibitor SB203580 (5 and 20 μM) reduced the promoter activity of MMP-1. SP600125 also reduced the gelatinolytic activity and MMP-1 protein expression in HLE cells. Taken together, JNK pathway is always activated in HLE cells, which results in constitutive expression of MMP-1.

Benbow *et al.*[38] reported that in melanoma cells, constitutive MMP-1 expression in the absence of the 2G single nucleotide polymorphism was mediated by p38 and ERK1/2 MAP kinases. Our data also support that, in the absence of 2G allele, HLE cells utilize the alternative activation mechanism to achieve high levels of MMP-1 expression.

Eferl *et al.*[14] have demonstrated that the requirement for c-Jun was restricted to the early stages of tumor (HCC) development by antagonizing p53 activity, resulting in suppression of apoptosis. JNK activation may be required for the early stages of HCC cells, because the activation leads the cells to be more invasive (through MMP-1 activation) and proliferative (via inactivation of p53). This activation seems to explain our previous findings showing that MMP-1 expression was restricted to the early stage of HCC. In addition, it is also reported that MMP-1 itself has an ability to induce hepatocyte proliferation.[13] Thus, these findings indicate the significance of not only c-Jun activation but also MMP-1 expression for the invasion and proliferation of HCC cells.

In summary, the present study indeed confirms the relationship between the JNK activation and MMP-1 expression in HCC cells and indicates the new aspect of c-Jun activation in the early stage of HCC development. Further investigations to clarify the difference in the regulatory mechanism of MMP-1 gene expression between regenerating hepatocyte and HCC cells will help us to reverse liver cirrhosis and to prevent HCC development by modifying MMP-1 expression.

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Costs of medical services for patients with HIV/AIDS in Khon Kaen, Thailand

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Objective: To estimate the savings and cost of providing highly active antiretroviral therapy (HAART) to adult patients with AIDS under Universal Coverage (UC) in Khon Kaen Province, Thailand.

Design: Micro-costing of outpatient and inpatient services of two referral hospitals, and cost modelling.

Setting: Khon Kaen Regional Hospital and Northeast Regional Infectious Hospital.

Patients: Adult patients who resided in Khon Kaen and made outpatient visits at and/or those who were discharged from those hospitals from 1 December 2001 to 28 February 2002.

Main outcome measure: The average cost per outpatient visit and per inpatient day. Based on these figures, the savings and cost of providing HAART to adult patients with AIDS under UC at outpatient settings in this province were estimated.

Results: The average cost per outpatient visit with and without antiretroviral drugs (ARV) was US$294.2 and US$26.1, respectively. The average cost per inpatient day with and without ARV drugs was US$368.1 and US$43.8, respectively. The net annual cost of HAART was estimated to be US$5 674 629. This is equivalent to 20.0% of the annual UC budget for adults in this province in 2002. Sensitivity analysis and projection to the year 2006 were conducted.

Conclusion: A large increase in the budget would be required to provide HAART to all adult patients with AIDS under UC. However, the sensitivity analysis showed it would be an affordable policy option if low-cost antiretroviral drugs were successfully introduced. This type of analysis would be useful to assess the financial implications of providing HAART in public health systems worldwide.

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Keywords: Thailand, AIDS, HIV, antiretroviral therapy, HAART, financial analysis

Introduction

The mortality rate from HIV has declined sharply and the quality of life of patients with HIV/AIDS has improved in developed countries since highly active antiretroviral therapy (HAART) was introduced in the late 1990s [1–3]. HAART has been a cost-effective intervention in developed countries [4–7], but its high cost inhibits many developing countries from providing such care through their public health care systems [8].

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Unequal access to treatment and adequate health services was one of the main reasons why there is a difference between the mortality rate for patients with HIV/AIDS in developed countries and those in developing countries [9]. While the prevention of HIV infection remains crucial, it is also important to address the need for adequate medical care, including HAART, among those who have already been infected with HIV [10–11].

Thailand is one of many countries facing the problems of HIV/AIDS. The number of people with HIV/AIDS was estimated to be 695 000, and 55 000 of them were expected to develop serious AIDS-related illnesses in 2000. It is also estimated that 2% of adult males and 1% of adult females are HIV positive [12].

Although Thailand established universal health insurance coverage (UC) in October 2001, HAART is not included in its benefit package. There is a huge demand to include HAART in the benefit package so that every patient with HIV/AIDS will have access to the therapy, regardless of their economic status. In response to this, the Public Health Minister, on the eve of National AIDS Day 2001, announced that HAART would be included in the UC benefit package in the future [13].

To prepare for implementation of such a decision, it is important to conduct an analysis of its financial feasibility, because providing HAART is still expensive. The objectives of this study are to estimate the cost of HIV/AIDS patient care at two public hospitals in Khon Kaen Province in Thailand and, based on the data collected, to estimate the savings and cost of providing HAART for adult patients with AIDS in the province.

Methods

Study site

Khon Kaen is one of the provinces in the northeast, an economically less-developed part of Thailand, and has often been chosen as a model province for new policies before the Ministry of Public Health (MOPH) implements them nationally. Its population was 1.75 million in 2001.

Data were collected at Khon Kaen Regional Hospital (KKRH) and Northeast Regional Infectious Hospital (NEIH). KKRH is a referral hospital for both the community hospitals in this province and the general hospitals in the neighbouring provinces. KKRH also functions as a community hospital for the residents in the vicinity. KKRH has a follow-up clinic for HIV-positive patients and provides admission services for them in the Department of Medicine. NEIH is a specialized hospital for infectious diseases, and its focus areas are 19 provinces in northeast Thailand. NEIH has an HIV clinic and an inpatient ward for the HIV-positive patients.

There are currently three major medical insurance schemes in Thailand: the Civil Servant Medical Benefit Scheme, the Social Security Scheme and the UC Scheme [14]. The first covers civil servants, employees of government enterprises and their family members; the second covers employees of private companies, and UC covers the rest of the population. All of them cover medical expenses for the treatment of opportunistic infections in those with HIV/AIDS. The Civil Servant Medical Benefit Scheme covers the expense of antiretroviral (ARV) therapy only when it is given as part of inpatient care.

At the time of the study, both KKRH and NEIH participated in the project on HAART under MOPH by which 63 patients in total received HAART.

Data collection

The subjects were patients with HIV/AIDS who resided in Khon Kaen Province and were 20 years old or over at the time of the study. Data were collected at each outpatient visit and included health insurance, laboratory tests, diagnosis, prescription and charges (laboratory tests and drugs). The length of stay was also collected for inpatients. Nurses in each department collected the data from the medical chart and prescription forms and entered them on the questionnaires that were prepared for each outpatient visit and admission. Charges for medicine and laboratory tests were calculated based on the fee schedules of the hospitals. All the information collected was coded by the hospital staff so that outside researchers could not identify individual patients. The study period was from 1 December 2001 to 28 February 2002. Inpatient data were collected from those who were discharged during the study period.

Analysis for the micro-costing of HIV/AIDS patient care

The average charges for medicines and laboratory tests per outpatient visit and inpatient day were calculated with and without ARV drugs by HIV stages. Of those who received ARV drugs, the average charges for ARV drugs per outpatient visit and hospital day were calculated by type of drug combination: one drug, two drug and HAART. In this study, HAART is defined as the regimen that combines three or more different antiretroviral drugs. For HIV stages, physicians in the hospitals grouped patients as asymptomatic HIV, with AIDS-related complex (ARC) or with clinical AIDS symptoms according to the 1993 US Center for Disease Control and Prevention staging system for HIV, which the Thai Government has adapted for its surveillance

case definition [15]. If it was not specifically mentioned in the medical chart, the stage was determined according to the diagnosis. For example, the patients with non-tuberculous pneumonia, diarrhoea or oral candidiasis were categorized as ARC, and those with cytomegalovirus or cryptococcus meningitis as AIDS.

Medicines and laboratory tests are charged at up to 15% above their procurement cost at public hospitals in Thailand. In this study, a cost-to-charge ratio of 1 was employed to estimate their cost and sensitivity analyses were conducted with the ratio of 0.85. To estimate the average medical costs per outpatient and inpatient day, with and without ARV drugs, the average costs of medicines, laboratory tests and services were summed up for routine outpatient and inpatient service, respectively. The routine outpatient and inpatient service costs include personnel, administrative and capital costs of these services and were available from the hospital cost analysis at KKRH in 1998 [16]. They were US$2.2 per outpatient visit and US$21.0 per inpatient day, which was adjusted to the 2002 price with the average annual medical service inflation rate of 3.0% [17]. The average costs per outpatient visit and inpatient day for those with and without HAART were estimated from the MOPH perspective [18].

Estimation of the number of people with HIV/AIDS in Khon Kaen Province

The number of adult patients with AIDS under UC in Khon Kaen (AAP) in 2002 was not directly available. The number of adult HIV-positive patients was estimated from the adult population in Khon Kaen Province in 2000 [19], the national HIV prevalence rate (males, 2%; females, 1%) [12] and the annual population growth rate of 1.1% [20]. The number of patients with AIDS was estimated based on the proportion of patients with AIDS to the HIV-positive population at the national level in 2002 (males, 11.3%; females, 9.4% [12]). It was assumed that the proportion of those covered by UC in the province would be the same as the proportion in our study.

Modelling of province-wide costs of antiretroviral therapy

It has been reported that the introduction of HAART reduced the spending on hospitalization in developed countries [1,2]. On the assumption that there would be savings through a reduction in hospitalization under UC in Khon Kaen Province if HAART was provided to all adult patients with AIDS, the following formula was used to calculate the savings and costs of such a strategy:

$$\text{Savings} = (\text{RIC} + \text{AMC}) \times \text{PRD} \times \text{THD}$$

where RIC is a routine inpatient service cost per day [17], AMC is the average cost of medicines and laboratory tests per inpatient day among the patients with AIDS without ARV drugs, PRD is the reduction in hospital days resulting from the provision of HAART to the patients with AIDS (taken to be a 40% reduction in bed days [21]), and THD is the estimated total annual hospital days of all adult patients with AIDS under UC. THD was estimated using the following formula:

$$\text{THD} = [\text{IP}/(\text{OP} + \text{IP}) \times \text{AAP}] \times \text{LOS} \times \text{AAR}$$

where IP is the number of current inpatients with AIDS under UC without ARV drugs, OP is the number of current outpatients with AIDS under UC without ARV drugs, LOS is the average length of stay for AIDS inpatients under UC without ARV drugs, and AAR is their annual admission rate (taken in this estimation as three times per person per year [22]). OP/(OP + IP) is the proportion of patients with AIDS requiring inpatient care.

The total annual cost (TAC) for providing HAART to the adult patients with AIDS under UC in Khon Kaen was calculated using the following formula:

$$\text{TAC} = [(\text{ROC} + \text{ADC}) \times 12 + 4\text{LTC}] \times \text{AAP} + \text{CIT}$$

where ROC is the routine cost per outpatient visit [17], ADC is the average cost of HAART per outpatient visit, LTC is the estimated cost of laboratory tests (CD4 cell count, viral load, complete blood count) based on the fee schedule of the hospital, and CIT is the estimated cost for in-service training in this province (MOPH Disease Control Office Region 6, Khon Kaen). The calculation of TAC assumed that (i) all patients with AIDS would receive HAART at an outpatient department, (ii) the patients would visit the outpatient department 12 times per year, (iii) laboratory tests would be done four times per year [23] and (iv) a 2-day training programme would be conducted to prepare physicians, pharmacists, nurses, counsellors and laboratory technicians at 23 public hospitals (20 community hospitals and three tertiary hospitals including KKRH and NEIH) in the province for providing HAART.

The estimated net costs for providing HAART for the patients with AIDS under UC in Khon Kaen in 2002 were calculated by subtracting the savings from TAC. This was compared with the estimated annual budget of UC in Khon Kaen Province. The annual budget for UC was calculated based on 80% of the adult population being covered by UC in Khon Kaen Province (Khon Kaen Provincial Health Office). The annual per

capita budget of UC for the fiscal year 2002 was taken as US$30.3 [24].

Sensitivity analysis

Sensitivity analyses were conducted to examine uncertainties in the cost modelling, and the proportion of the net costs to the UC budget in this province was projected to 2006. Uncertainties examined in the analysis were: cost of inpatient care lower by 20%; the proportion requiring inpatient care among AIDS higher by 25%; cost-to-charge ratio 0.85; HIV prevalence rate lower by 25%; cost of HAART US$325.0 per year, the cost of GPO-VIR (a domestically produced combination antiretroviral pill [25]); the number of laboratory tests two times per year. The projection of the proportion of annual net cost of HAART to the UC budget contained the following assumptions: (i) the survival rate of patients with AIDS with HAART for 12 months would be 80%; (ii) Khon Kaen population would annually grow by 1.1%; (iii) the number of new AIDS cases in each year would change by the same proportion as the national projection of the baseline scenario [12]; (iv) the annual inflation rate would be 3.0%; (v) the UC budget per person would grow by 4.5% annually, the baseline projection of annual economic growth rate [26].

Thai bahts were converted into US dollars at an exchange rate of US$1 to 39.71 Thai baht.

Results

Patient characteristics

There were 552 outpatient visits. The number by HIV stages was: asymptomatic HIV 66 (11.9%), ARC 247 (44.7%), AIDS 237 (42.9%), and missing two (0.4%). There were 200 inpatients. The number by HIV stages was: asymptomatic HIV 16 (8.0%), ARC 40 (20.0%), AIDS 143 (71.5%), and missing one (0.5%). In total, 312 (82.1%) of the patients with AIDS were covered by UC.

Average costs per HIV/AIDS patient

Table 1 shows the average charges per outpatient visit and inpatient day with and without ARV drugs by medicine, laboratory test and HIV stages. By adding the routine service costs to these figures, the estimated average costs per outpatient visit with and without ARV drugs were US$294.2 and US$26.1, respectively. The estimated average costs per inpatient day with and without ARV drugs were US$368.1 and US$43.8, respectively.

Province-wide cost of antiretroviral therapy

Table 2 shows the savings and TAC of providing HAART for patients with AIDS under UC in outpatient settings in 2002. Savings and TAC were estimated to be US$194 813 and US$5 869 442, respectively. The net cost was US$5 674 629. This amount is equivalent to 20.0% of the estimated UC budget for adults in Khon Kaen in fiscal year 2002.

Sensitivity analysis and projection

Table 3 shows the results of the sensitivity analysis and the projection of net cost of HAART for the adult patients with AIDS under UC in Khon Kaen Province. Changing the current regimen to GPO-VIR would reduce the proportion of province-wide cost of HAART to the UC budget to 3.7% in 2002, an 80% reduction. If HIV prevalence was lower by 25%, the proportion would be 14.9%, a 26% reduction. Projection shows that the proportion in the baseline would be 45.4% in 2006. It is also projected that the

Table 1. Average charges for patients with HIV/AIDS by medicine, laboratory test and HIV stage.[a]

	Outpatient (US$/visit)		Inpatient (US$/inpatient day)	
	ARV (n = 106)	No ARV (n = 444)	ARV (n = 35)	No ARV (n = 154)
Total	292.0	23.9	347.1	22.8
Medicine	289.2	20.5	328.3	7.7
ARV	279.8	n.a.	297.6	n.a.
Two drugs[b]	154.1	n.a.	n.a.	n.a.
HAART	281.0	n.a.	297.6	n.a.
Laboratory test	2.9	3.4	18.8	6.1
HIV stages				
Asymptomatic	297.3	9.5	486.3	n.a.
ARC	275.9	15.8	263.4	19.6
AIDS	324.1	34.9	305.6	24.5

ARV, antiretroviral therapy; HAART, highly active antiretroviral therapy (three or more drugs); ARC, AIDS-related complex; n.a., not available.
[a]Two outpatient visits and 11 admitted patients without complete data were excluded from this analysis.
[b]One case only.

Table 2. Estimated cost of providing highly active antiretroviral therapy to adult patients with AIDS under Universal Coverage in Khon Kaen Province in 2002.[a]

Savings	
Estimated total savings (US$)	194 813
Routine inpatient service cost per day (RIC, US$)	21.0
Average cost of medicines and laboratory tests per inpatient day among the patients with AIDS without ARV (AMC, US$)	24.5
Estimated total annual hospital days (THD[b], days)	10 704
Proportion of reduction in hospital days (PRD, %)	40.0
Cost	
Estimated total annual cost (TAC, US$)	5 869 442
Routine outpatient service cost per visit (ROC, US$)	2.2
Average drug cost per visit with HAART (ADC, US$)	281.0
Number of visits per year	12
Laboratory tests costs per visit (LTC, US$)	114.8
Laboratory tests per year	4
Estimated number of adult patients with AIDS under UC in Khon Kaen (AAP, persons)	1520
Cost for inservice training of the medical personnel (CIT, US$)	5890

[a]See Methods for calculation of the values given.
[b]THD = [106/(106 + 183) × 1520] × 6.4 × 3.

proportion would be 8.3% in 2006 if GPO-VIR replaces the current regimen.

Discussion

This study attempted to estimate savings and cost of providing HAART to adult patients with AIDS under UC in a rural province in Thailand, based on the cost of care for the patients with HIV/AIDS who were treated at two tertiary public hospitals in the province.

There are a limited number of cost studies of the medical care for people with HIV/AIDS in developing countries. In our study, the average cost per inpatient day was US$43.8 for the patients with AIDS without ARV drugs. This is higher than the findings from tertiary hospitals in African countries (US$18.0–40.0) [27,28].

The financial analysis in this study indicated that a substantial increase of resources would be necessary to provide HAART to adult patients with AIDS under UC in this province; however, the study had several limitations. First, the average inpatient cost per day in the study could be higher than that in the province as a whole. The proportion of severe cases in our study sample might be higher than those in other public hospitals because our data were collected from referral hospitals. Second, the proportion requiring inpatient care may be underestimated because the same person could be counted twice, in both outpatient and inpatient data. Such patients could not be identified because the data were anonymous. Third, the number of adult patients with AIDS in this study could be overestimated because it was estimated from the national figures. It is likely that the prevalence at the national level is higher than that of Khon Kaen because other regions are known to have prevalence rates much higher than the national figure [29]. Finally, the Government Pharmaceutical Organization in Thailand started producing GPO-VIR a few months after the end of the study; this combination pill costs about one-tenth of the triple therapy that was available at the time the study [30]. It is expected that the introduction of

Table 3. Sensitivity analysis and projection of net cost of highly active antiretroviral therapy for adult patients with AIDS under Universal Coverage (UC) in Khon Kaen.

	Percentage added to the UC budget for the adults	
Uncertainties examined	2002	2006
Baseline[a]	20.0	45.4
Inpatient cost lower by 20%	20.2	45.7
Proportion requiring inpatient care higher by 25%	19.9	45.1
Cost-to-charge ratio 0.85	17.0	38.6
HIV prevalence rate lower by 25%	14.9	33.7
GPO-VIR (US$325 per year)	3.7	8.3
Laboratory test 2 times a year	18.8	42.6

GPO-VIR, a combination antiretroviral pill manufactured in Thailand.
[a]Calculated from the results in Table 2.

this new drug would greatly improve the financial feasibility of providing HAART.

These uncertainties were examined in the sensitivity analysis, which showed that the net cost of providing HAART could be much lower than the our initial estimation if GPO-VIR can replace the current regimen. However, even with 90% reduction in drug cost, the estimated cost of HAART provision was still larger than the estimated savings from a reduction in hospitalization. This is contrary to studies in developed countries [1,2]. This might be because of the differences in the labour cost between Thailand and the developed countries. Hospitalization in a developing country like Thailand may be relatively inexpensive compared with that in the developed countries [31,32].

The net cost of HAART is projected to be equivalent to nearly half of the UC budget in this province in 2006. This indicates that adding HAART to the UC benefit package would be prohibitively expensive and may not be feasible in terms of sustainability. However, if GPO-VIR could successfully replace the current regimen, the proportion would be 8% in the same year. This proportion may be within the range manageable by MOPH. Although the effectiveness of GPO-VIR needs to be carefully monitored, if it is as effective as other ARV drugs and adequate amounts can be supplied at the current low costs, adding HAART to the UC benefit package may be a feasible option.

Indeed, providing HAART in its public health system might become a realistic policy option for many developing countries in the near future, as it is in Thailand, if low-cost ARV drugs such as GPO-VIR become widely available. In such situations, it is envisaged that the kind of analysis that we conducted in this study would be useful to assess the financial implications of providing HAART in the public health system in each country.

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