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Virology 369 (2007) 198–205

VIROLOGY

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Up-regulation of hepatitis C virus replication by human T cell leukemia virus type I-encoded Tax protein

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Received 22 June 2007; returned to author for revision 18 July 2007; accepted 31 July 2007
Available online 4 September 2007

Abstract

Co-infection of hepatitis C virus (HCV) with other blood-borne pathogens such as human T cell leukemia virus (HTLV) is common in highly endemic areas. Clinical evidence showing a correlation between HTLV-I co-infection and rapid progression of HCV-associated liver disease promoted us to investigate the effect of HTLV-I-encoded Tax protein on HCV replication. Reporter assay showed that HCV replicon-encoded luciferase expression was significantly augmented by co-transfection of the Tax-expressing plasmid. Further, HCV RNA replication in replicon cells was increased either by co-culture with cells stably expressing Tax protein (Huhtax) or by culture in the presence of Huhtax-conditioned medium, indicating that Tax could also modulate HCV replication of adjacent cells in a paracrine manner. Additionally, HCV replication in Huhtax exhibited a reduced responsiveness to interferon-α-induced antiviral activity. This study demonstrates the facilitation of HCV replication by Tax protein, which may partially account for severer clinical consequences of HCV-related disease in HCV/HTLV co-infected individuals.
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Keywords: HCV; HTLV-I; Co-infection; Replication

Introduction

Hepatitis C virus (HCV) is the major causative agent of posttransfusional non-A, non-B viral hepatitis. Chronic HCV infection is frequently associated with liver cirrhosis and hepatocellular carcinoma (HCC), being a global health threat and the main cause of adult liver transplants in developed nations. HCV is a member of the Flaviviridae family with a positive-sense RNA genome of ~9600 nucleotides in length (Choo et al., 1989). The genome is flanked by highly structured nontranslated regions (NTRs) important for both RNA translation and replication. The viral genome encodes a polyprotein precursor of approximately 3010 amino acids, which is processed by viral and cellular protease to produce the structural proteins (core, E1 and E2) and nonstructural (NS) proteins (p7 and NS2 to NS5B) (Major and Feinstone, 1997).

Human T cell leukemia virus type I (HTLV-I) has been etiologically linked to the development of adult T cell leukemia/lymphoma (ATL) (Yoshida et al., 1984) and to chronic conditions including tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM) (Gessain et al., 1985; Osame et al., 1986). The HTLV-I genome encodes several regulatory proteins including Tax that are essential for viral replication. In addition to transcriptionally activating the promoter in 5′ long terminal repeat, Tax has also been shown to regulate the transcriptional activity of cellular promoters by serving as a transcriptional cofactor for the cyclic AMP-responsive element-binding factor (CREB), NF-κB, and the serum-responsive factor (SRF) pathways. Most of the cellular genes modulated by Tax are involved in growth, differentiation, apoptosis and cell cycle control.

HCV/HTLV-I co-infection is frequent in the regions that are highly endemic for both viral infections, probably due to the

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doi:10.1016/j.virol.2007.07.032

similarity of the transmission route. Multiple lines of clinical evidence have shown that HCV co-infection with HTLV-I is associated with an increased risk of progression to severe liver disease and an elevated mortality due to liver cancer (Boschi-Pinto et al., 2000; Kishihara et al., 2001), highlighting the involvement of HTLV-I in HCV-related pathogenic processes. Although it is generally believed that the immunosuppressive effects of HTLV-I may exacerbate the progression of liver disease by attenuating the cytotoxic T lymphocyte response to HCV-infected hepatocytes, molecular interaction between HCV and HTLV-I might also play a role in the pathogenesis of severe liver disease.

In this study, we investigated possible interaction between HCV and HTLV-I. Our data indicate that HTLV-I-encoded Tax protein up-regulates the replication of HCV RNA in replicon cells and the Tax-mediated HCV activation was observed not only in Tax-expressing cells themselves but in those neighboring exposed cells as well.

Results

Activation of HCV replication by Tax

To investigate the possible influence of Tax protein on HCV replication, a human hepatoma cell line (Huh-7) was transfected with Tax-expressing plasmid (pCnwtax), and HCV replicon RNA in vitro transcribed from pLMH14, which contains the 5′ NTR, the first 36 nucleotides of the core region fused directly with the firefly luciferase reporter gene, the IRES element from encephalomyocarditis virus (EMCV) that directs translation of the HCV proteins from NS3 to NS5B and the 3′ NTR of HCV RNA (Fig. 1A) (Murata et al., 2005; Zhang et al., 2005b). The replicon-encoded luciferase expression, which was used as a read out of HCV RNA levels, was assayed at 3 and 72 h post-transfection. The luciferase levels at 3 h posttransfection were used to normalize the transfection efficiency. As shown in Fig. 1B, the luciferase expression from LMH14 replicon was sig-

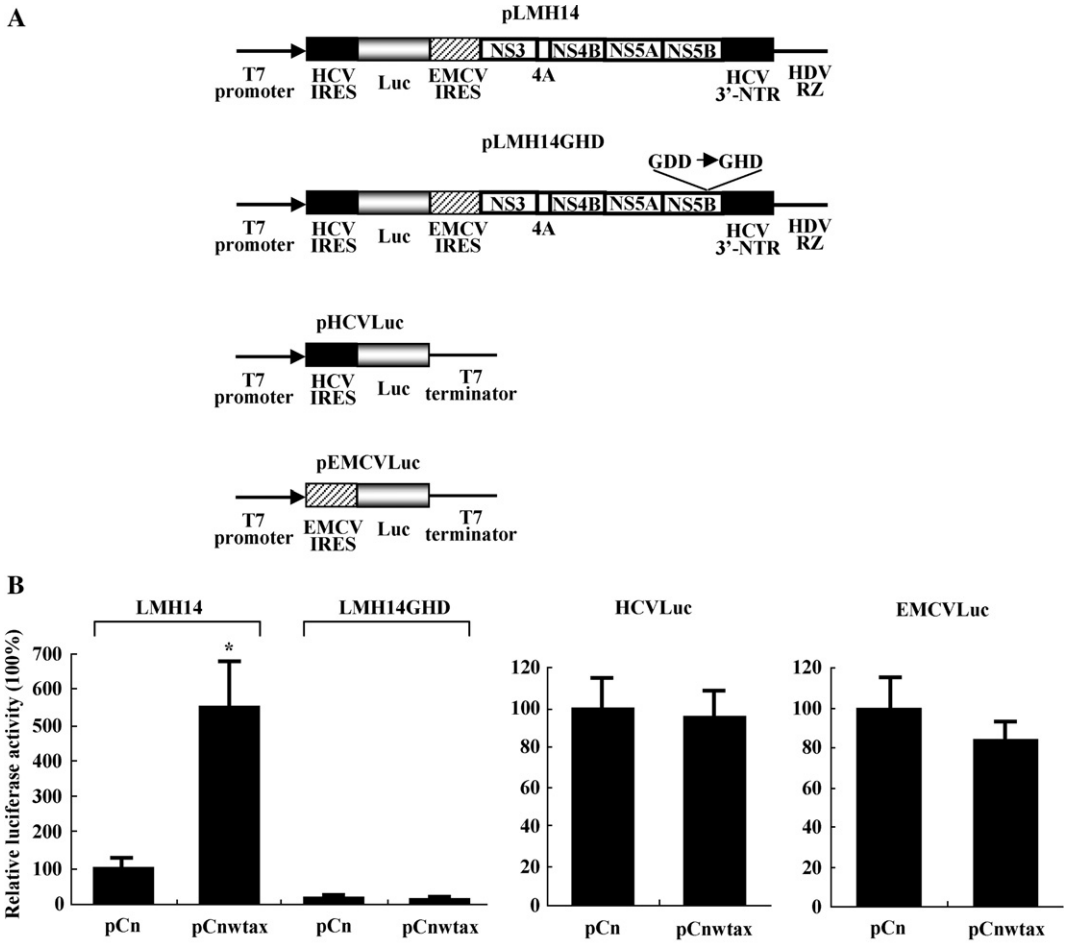

Fig. 1. Activation of HCV replication by HTLV-I Tax. (A) Schematic diagrams of the HCV replicon construct pLMH14, the replication-deficient replicon pLMH14GHD, as well as two control reporter vectors pHCVLuc and pEMCVLuc. pLMH14 contains T7 promoter, 5′ nontranslated region (NTR), the first 36 nucleotides of the core region fused directly with the firefly luciferase reporter gene, the internal ribosome entry site (IRES) element from encephalomyocarditis virus (EMCV) that directs translation of the HCV proteins from nonstructural (NS)3 to NS5B and the 3′ NTR of HCV RNA. pLMH14GHD is identical to pLMH14 except for carrying an inactive GHD motif in RdRp. pHCVLuc and pEMCVLuc contain the luciferase gene downstream of T7 promoter and HCV or EMCV IRES elements identical to that inserted in pLMH14, respectively. (B) The RNAs in vitro transcribed from the indicated vectors were transfected into Huh-7 cells together with pCn or pCnwtax-expressing Tax protein. Luciferase activities in the lysates were measured at 3 h and 72 h posttransfection. Luciferase activities at 3 h were used to normalize the transfection efficiency. Relative luciferase activity at 72 h in cells transfected with pCn was defined as 100%, and that in pCnwtax transfectant is expressed as relative percentage. The results are from five independent triplicate transfections. Luc, luciferase. *$P<0.05$ compared with pCn.

nificantly enhanced in cells expressing Tax protein, being approximately five- to six-fold of that in cells transfected with the empty vector pCn (previously referred to as pCMV-NEO-BAM in Baker et al., 1990), whereas co-transfection of pCnwtax did not significantly affect the luciferase expression from replication-deficient replicon LMH14GHD, in which the catalytic GDD motif of NS5B polymerase was replaced by inactive GHD motif (Fig. 1A). In view of the structural characteristic of pLMH14, in which the luciferase gene is synthesized under the control of HCV IRES and the NS proteins are initiated by EMCV IRES, thus up-regulation of HCV IRES- and/or EMCV IRES-mediated translation may also lead to an enhanced luciferase expression from LMH14 replicon. To investigate whether the augmentation of luciferase expression by Tax was due to its influence on HCV IRES- or EMCV IRES-dependent translation, we performed reporter assays with the RNA in vitro transcribed from reporter vectors pHCVLuc and pEMCVLuc (Fig. 1A), in which the expression of reporter gene is directed by HCV IRES and EMCV IRES elements identical to those inserted in pLMH14, respectively. Neither HCV IRES- nor EMCV IRES-directed luciferase expression was significantly affected by co-transfection of pCnwtax, indicating that the observed increment in luciferase expression was due to an enhancing effect of Tax on HCV replication rather than to its influence on HCV IRES- or EMCV IRES-mediated translation. Taken together, these results suggest that HTLV-I Tax protein enhances the replication of HCV replicon.

The molecular mechanism underlying Tax-mediated HCV activation

Tax protein has been shown to exert its biological activities through distinct pathways: it activates HTLV-I transcription through CREB/ATF, induces transcription of HIV, IL-2, IL-2Rα, GM-CSF and expression of the cellular immediate early genes via NF-κB and SRF pathways, respectively. To explore the molecular mechanism underlying Tax-mediated activation of HCV replication, two Tax mutant-expressing plasmids, pCnm148 and pCnm319, were employed in transient transfection experiments (Yamaoka et al., 1996). m148 (G148V) retains the ability to activate CREB/ATF pathway while failing to activate NF-κB pathway. Conversely, m319 (L319R and L320S) was shown to be functional in activating NF-κB pathway while lacks the ability to activate CREB/ATF pathway. As expected, reporter gene expression under the control of HTLV-I LTR promoter was enhanced substantially by co-transfetion with plasmid expressing wild-type Tax (wtax) or m148, but only moderately by m319 (Fig. 2A, upper), on the other hand, HIV-I LTR-directed luciferase expression was significantly increased by co-transfetion with plasmid expressing wtax or m319, but not by m148 (Fig. 2A, middle).

To compare the effects of wtax, m148 and m319 on HCV replication, plasmid expressing wild- or mutant-type Tax was transfected into Huh-7 cells together with the replicon RNA in vitro transcribed from pLMH14, and the replicon-encoded luciferase activities were measured as described above. As

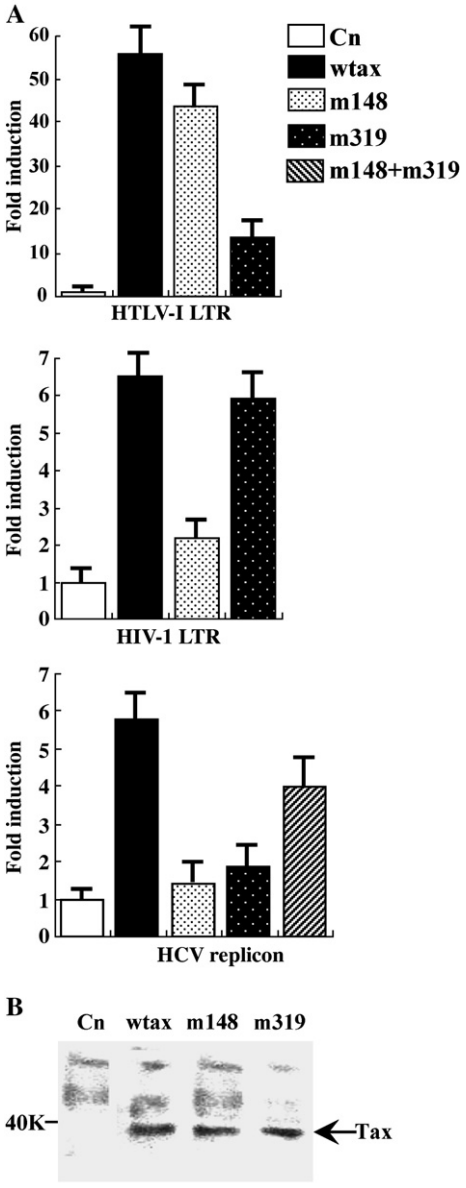

Fig. 2. Effect of Tax or its mutants on HCV replication. (A) Huh-7 cells were transfected with pHTLVLTR-Luc (upper), pHIVLTR-Luc (middle) or the replicon RNA in vitro transcribed from pLMH14 (lower), along with pCn, pCnwtax, pCnm148 or pCnm319. Relative luciferase activities were determined as described for Fig. 1. Fold induction means the luciferase activity relative to that co-transfected with the empty vector pCn. The results are from four independent triplicate experiments. (B) Expression of Tax protein in each transfectant was confirmed by Western blot analysis. Solid arrow indicates the signals of Tax protein.

shown in Fig. 2A (lower), Tax-mediated enhancement of HCV replication was significantly abrogated either by mutation incorporated in m148 or those in m319. Interestingly, co-expression of m148 and m319 restored the replicon-encoded reporter gene expression to some extent. This result suggests that both NF-κB- and CREB-dependent pathways may be involved in Tax-mediated activation of HCV RNA replication. In these experiments, expression of wild- or mutant-type Tax protein was confirmed by Western blot analysis, and the representative result is shown in Fig. 2B.

The paracrine effect of Tax protein on HCV replication in adjacent cells

While a growing body of evidence has shown that HCV can replicate efficiently in extrahepatic tissues and cells including peripheral blood mononuclear cells, there is little demonstration of HTLV-I infection in hepatic cells. It is thus not certain whether a scenario of co-infection of HTLV-I and HCV in same hepatic cell does occur in dually infected individuals. It has been shown that Tax protein is released from HTLV-I-infected and Tax-transfected cells and exerts its biological activities in neighboring exposed cells (Marriott et al., 1992; Cowan et al., 1997; Szymocha et al., 2000). Further, on the basis of the observation with BHK-21 and 293T cells transiently expressing Tax protein, Alefantis et al. (2005) reported that Tax is released into the extracellular environment by cellular secretion process other than apoptosis or lysis of the cells. In view of these considerations, we next investigated whether Tax protein released from certain HTLV-I-infected cells could modulate HCV replication of adjacent cells in a paracrine manner. MT-2 cells provide a tool to study the effect of paracrine-acting Tax, however, a lesson obtained from studying the molecular interaction between HTLV-I and HIV showed that MT-2 supernatant additionally contains multiple factors with adverse effect on HIV infection (Moriuchi et al., 1998), which makes it difficult to investigate the authentic effect of soluble Tax. Accordingly, instead of MT-2 cells, we employed Huh-7 cells stably expressing wild- or mutant-type Tax protein for this purpose. Huh-NNRZ cells, a Huh-7-derived cell line constitutively replicating HCV subgenomic replicon (Kishine et al., 2002; Zhang et al., 2004), were co-cultured with HuhCn, Huhwtax, Huhm148 or Huhm319 cells, which were established by transfection with pCn, pCnwtax, pCnm148 or pCnm319 followed by G418 selection. Similar level of Tax expression in each cell line was confirmed by Western blot analysis (Fig. 3A). Three days later, total RNAs were extracted and subjected to real-time RT-PCR for quantification of HCV replicon RNA levels. As shown in Fig. 3B, HCV RNA was significantly increased in Huh-NNRZ cells co-cultured with Huhwtax, being approximately 6-fold higher than that co-cultured with HuhCn, while co-culture with Huhm148 or Huhm319 did not obviously affect the HCV RNA level in replicon cells. Similar result was also obtained when Huh-NNRZ cells were incubated in the presence of 50% supernatant from Huhwtax cell culture (Fig. 3C). Moreover, pretreatment with anti-Tax antibody reduced, although did not abolish completely, the ability of Huhwtax-conditioned medium to activate HCV replication, confirming a specific role of Tax in the enhanced HCV replication. The fact that the stimulatory activity was partially retained even after treatment with anti-Tax antibody may be attributable to the enhancing effect of other soluble factors pre-induced by Tax in the conditioned medium. Indeed, it was reported that the activation of CMV replication by Tax protein is largely mediated by induction of interleukin-8 and transforming growth factor-β (Szabo et al., 1999).

To further confirm that Tax protein could affect HCV replication in a paracrine manner, we next examined the effect of

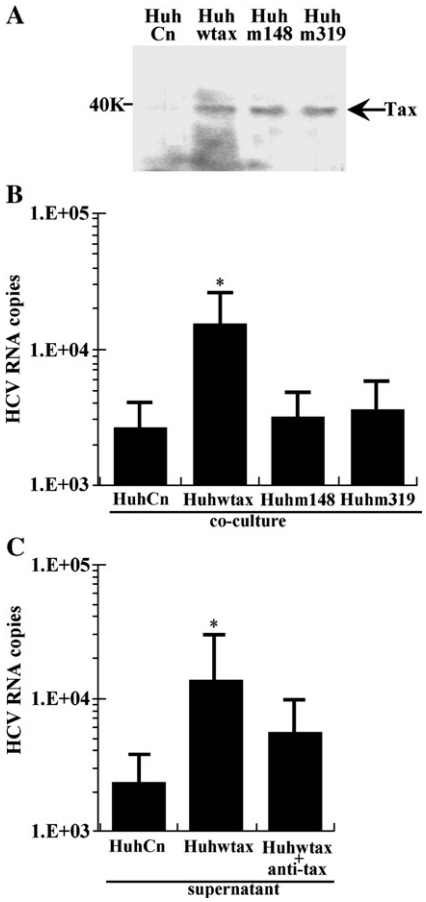

Fig. 3. Paracrine effect of HTLV-1 Tax on HCV replication. (A) Similar level of Tax expression in cell lines stably expressing wild- or mutant-type Tax. Huh-7 cells were transfected with pCn, pCnwtax, pCnm148 or pCnm319 followed by G418 selection, and Tax expression was detected by Western blot analysis. Huh-NNRZ cells were co-cultured with the indicated stable cell lines (B) or were incubated in the presence of 50% culture supernatants from HuhCn or Huhwtax pretreated with or without anti-Tax (C). After a 3-day culture, the cells were harvested and HCV replicon RNAs were quantified with real-time RT-PCR. GAPDH mRNA level in each sample was simultaneously quantified to normalize the value of HCV replicon RNA. Representative data are from three separate experiments. *$P<0.05$ compared with HuhCn.

recombinant Tax protein expressed in *Escherichia coli* on HCV replication. GST-Tax was purified and added to the culture of Huh-7 cells transfected with HCV replicon RNA at 3 h posttransfection. After an additional 72 h, the replicon-encoded luciferase activities were measured and corrected by those determined at 3 h posttransfection. As shown in Fig. 4B, incubation of Huh-7 cells in the presence of 5 nM GST-Tax protein, but not of GST protein, resulted in an enhanced replication of HCV replicon. Together with the results described above, the data demonstrate that Tax protein could also up-regulate HCV replication in neighboring exposed cells.

Modulation of interferon-α-induced antiviral activity by Tax

Next we were interested in investigating whether Tax alters the responsiveness of HCV replication to interferon (IFN)-α. To this end, Huhwtax and HuhCn cells were transfected with the replicon RNA in vitro transcribed from pLMH14. Cells were

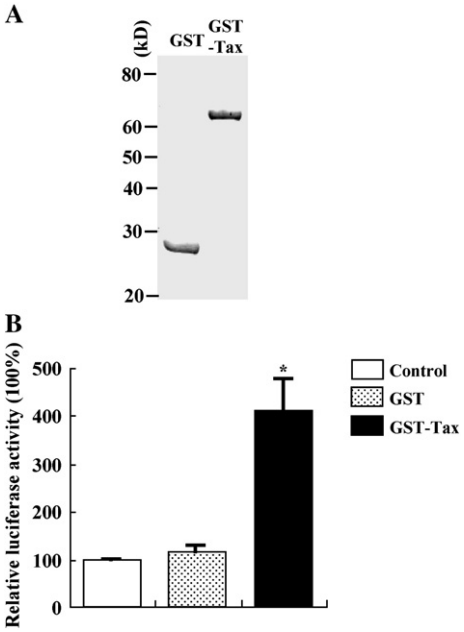

Fig. 4. Up-regulation of HCV replication by recombinant Tax protein. (A) SDS-PAGE analysis of purified GST and GST-Tax proteins. The gel was stained with Coomassie blue. (B) Huh-7 cells transfected with replicon RNA was cultured in the absence or presence of 5 nM GST, or GST-Tax for 72 h, and the relative luciferase activities in the lysates were determined as described for Fig. 1. The results are from two independent triplicate transfections. *$P<0.05$ compared with control.

harvested and divided into several aliquots, which were further incubated for 3 days in the presence of various IFN-α concentrations ranging from 0.3 to 100 IU/ml. The corrected replicon-encoded luciferase activities were shown in Fig. 5A. At each IFN concentration, luciferase activity detected in Huhwtax was significantly higher than that in HuhCn, and the difference was more significant with the increase of IFN concentration. Although a similar dose-dependent manner was observed, the IFN-α responsiveness of HCV replication in Huhwtax cells was poorer than that in HuhCn cells, with a 50% inhibitory concentration (IC_{50}) of about 3 to 10 IU/ml and 10 to 30 IU/ml, respectively.

Additionally, it was reported that HCV replication was highly dependent on cellular proliferation, both viral RNA and protein syntheses were largely increased in actively growing cells (Pietschmann et al., 2001; Scholle et al., 2004). To investigate whether the stimulatory effect of Tax on HCV replication was mediated by its influence on cell proliferation, growth characteristics for Huhwtax and HuhCn cells were analyzed. Expression of Tax protein did not significantly affect cell growth of Huh-7 cells, as evidence by similar growth curve was delineated for Huhwtax to that for HuhCn (Fig. 5B). This observation thus rules out the possibility that Tax up-regulates HCV replication via stimulating host cell growth.

Discussion

In this study, we have demonstrated that HTLV-I Tax protein up-regulates HCV replication, and this activation is not only limited in Tax-expressing cells but extended to those exposed neighboring cells. The latter is probably more important, considering that it is not certain whether co-infection of HTLV-I and HCV in same hepatic cells is possible in vivo because hepatic cells are not known as typical target cells for HTLV-1. With this respect, results presented in Figs. 1 and 2 may be of less clinical relevance because the experimental setting required co-infection of HTLV-1 and HCV in same target cells, however, these results provided important mechanistic information, which promoted us to further investigate the paracrine effect of Tax on HCV replication. Tax has been shown to be released from infected cells, and extracellular Tax has been reported to play pathological roles in multiple HTLV-1-associated diseases. For example, it was demonstrated that extracellular HTLV-I Tax activated the expression of endogenous IL-2Rα in lymphocyte, being a causative factor of the abnormal lymphocyte proliferation observed in ATL and TSP (Marriott et al., 1992). Also, it was reported that extracellular Tax induced TNF-α expression in neuronal cells (Cowan et al., 1997) and impaired the ability of astrocytes to manage the steady-state level of glutamate (Szymocha et al., 2000), both of which may contribute to the pathogenesis of HTLV-1-associated TSP. We

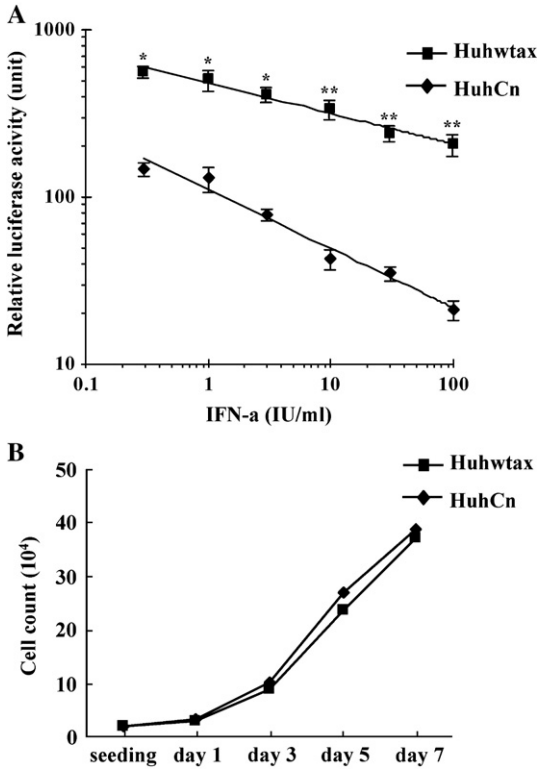

Fig. 5. (A) Modulation of interferon (IFN)-α responsiveness of HCV replication by HTLV-I Tax. HuhCn or Huhwtax cells were transfected with the replicon RNA in vitro transcribed from pLMH14, harvested and divided into several aliquots at 3 h posttransfection, which were further incubated for 3 days in the presence of various IFN-α concentrations ranging from 0.3 to 100 IU/ml. Replicon-encoded luciferase activity was measured and corrected by that determined at 3 h posttransfection. Representative results from three independent experiments are shown. *$P<0.05$ **$P<0.01$ compared with HuhCn. (B) Expression of Tax does not affect cell growth. HuhCn or Huhwtax cells were seeded at 2×10^4 cells per well in 24-well plates and were counted on the following days as indicated.

now extended this list by showing up-regulating effect of extracellular Tax on replication of co-infected HCV, which may consequently contribute to the pathogenesis of severe HCV-related diseases in HCV/HTLV co-infected individuals. However, to make the conclusion more physiologically relevant, one issue remaining to be addressed is whether the Tax protein secreted from hepatic cells is same as that from naturally infected T cells. Further investigation is under way to clarify whether the Tax protein secreted from infected T cells exerts a similar biological behavior in stimulating HCV replication. Additionally, it was demonstrated that extracellular Tax protein enhanced HIV-1 fusion/entry, probably by transactivating the expression of cellular co-receptors such as CXCR4 and CCR5 (Moriuchi et al., 1998). The subgenomic replicon used here constructively lacks the core to NS2 region, making it impossible to study the complete HCV life cycle including entry, assembly and release of viral particles. Thus, the effect of Tax on HCV replication in vivo may be more complicated than those observed here.

Several pathogenic viruses, such as HIV (De Rossi et al., 1991; Siekevitz et al., 1987), CMV (Moch et al., 1992; Szabo et al., 1998; Toth et al., 1995), SV40 (Nakamura et al., 1989) and JC viruses (Okada et al., 2000), have been reported to be activated by Tax through transcriptional activation of viral genes. HCV is a positive-stranded RNA virus, fulfilling its replication process in cytoplasm. So it is apparent that Tax activates HCV replication via a distinct mechanism, probably by transactivating the expression of cellular genes involved in HCV replication and/or by induction of 'pro-HCV' factors providing favorable circumstance for viral replication. Consistent with this hypothesis, it was found that treatment of Huhtax-conditioned medium with anti-Tax antibody did not fully abolish its stimulatory effect on HCV replication, the partially retained enhancing effect may be attributable to the pre-existing Tax-induced soluble factors in the culture supernatant. Whether mediated directly or indirectly by other factors, Tax, especially the paracrine-acting Tax, may play an important role in the pathogenesis of severe HCV-related disease in patients dually infected with HCV and HTLV-I. We showed by reporter assay that the ability of Tax to stimulate HCV replication was abrogated in Tax mutants incompetent to activate either NF-κB- or CREB-dependent pathway, and co-expression of these two mutants, however, largely restored the HCV activation by Tax. These results suggest that the downstream effective factors of these two signaling pathways may function in concert with each other in participating in Tax-induced HCV activation. Alternatively, the stimulatory effect of HTLV-I Tax on HCV replication may be mediated by a single factor which is transactivated by Tax via concurrently acting on NF-κB and CREB-responsive elements. Indeed, it was reported that both NF-κB and CREB pathways are essential for significant activation of anti-apoptotic gene *Bcl-xl* in human cells (Mori et al., 2001). Further experiments are required to distinguish these possibilities.

In addition to its direct activation of HCV replication, Tax may also contribute to rapid progression of HCV-associated liver disease by modulating the antiviral activity of IFN-α. Compared with that in HuhCn, a lower IFN responsiveness of HCV replication was observed in Huhwtax stably expressing Tax (Fig. 5A), which may provide a molecular basis for the clinical observation showing a significantly lower rate of sustained IFN response in HCV patients with concomitant HTLV-I infection than those infected with HCV alone (Kishihara et al., 2001). The precise mechanism for the influence of Tax on IFN responsiveness is currently unknown. When tested in a reporter assay with ISRE *cis*-luciferase vector, co-transfection of pCnwtax inhibited IFN-α-induced activation of ISRE promoter (authors' unpublished data). It was demonstrated that adenovirus E1A oncoprotein suppresses Jak-STAT pathway by interacting with CBP/p300 co-activators and consequently inhibiting the recruitment of them to STAT proteins (Look et al., 1998). Tax also utilizes CBP/p300 co-activators for implementing its transcriptional activation competence, it is thus conceivable but remains to be proven that competition between Tax and STAT1 for CBP/p300 binding may be responsible for the attenuated Jak-STAT signaling pathway by Tax protein. If this is the case, exogenous expression of CBP/p300 would restore the reduced IFN responsiveness by Tax. Additionally, it was reported that IFN-induced protein kinase PKR could phosphorylate HIV-1 Tat, and Tat inhibited the activity of PKR both by blocking its ability to autophosphorylate in response to dsRNA and by competing with its natural substrate eIF2 (Brand et al., 1997). Both Tat and Tax activate NF-κB pathway by stimulating the phosphorylation and degradation of IκB, which has been shown to be one of the substrates of PKR; thus such a mutual interaction may also be possible between Tax and PKR. Studies are under way to further define the molecular mechanism for Tax-conferred IFN-resistance.

Additionally, it has been shown that inclusion of the fusion protein (GST) to the N-terminal of foreign gene may interfere with the biochemical activity of some recombinant protein, probably by spatial hindrance. For example, it was reported that fusion of GST at the N-terminal of the eukaryotic DNA-binding protein, RSRFC4, could not be tolerated (Sharrocks, 1994). The GST-Tax used here, however, was shown to be biologically active, as evidenced by the data revealed in Fig. 4 and those reported by Moriuchi et al. (1998) and Moriuchi and Moriuchi (2006), by whom the pGST-Tax plasmid was kindly provided.

We investigated here the molecular interaction between HCV and HTLV-I, and provided evidence demonstrating that HTLV-I-encoded Tax protein up-regulates replication of HCV. Our findings suggest that HTLV-I may accelerate the clinical progression of HCV-related disease in dually infected patients by Tax-mediated enhancement of HCV replication, although dysfunction of cellular immune response by HTLV-I may also play a role.

Materials and methods

Plasmids

Parental plasmid pCn is identical to pCMV-NEO-BAM described elsewhere (Baker et al., 1990). Plasmids encoding

wild-type HTLV Tax (pCnwtax) or its mutants (pCnm148 and pCnm319) were described in the previous report (Yamaoka et al., 1996).

Cells

The cell line Huh-7 was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 U/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. The cell lines Huhwtax, Huhm148 and Huhm319, which stably express wild- or mutant-type Tax protein, were established by transfecting Huh-7 cells with pCnwtax, pCnm148 or pCnm319, followed by selection in the presence of 600 μg/ml G418 (Geneticin, Invitrogen). A Huh-7-derived cell line (Huh-NNRZ) stably replicating HCV subgenomic replicon was grown in DMEM medium containing 300 μg/ml G418 (Kishine et al., 2002; Zhang et al., 2004).

Preparation of recombinant Tax protein

GST-Tax fusion protein was induced by isopropyl-thiogalactopyranoside in *E. coli* DH5a cells transformed with pGST-Tax (Moriuchi et al., 1998), purified by binding to glutathione-Sepharose 4B (Bulk GST Purification Module, GE Healthcare), and recovered by elution with 10 mM reduced glutathione.

In vitro transcription

For synthesis the replicon RNA, pLMH14 or pLMH14GHD was linearized at *Xba*I site located immediately downstream of the HDV ribozyme, and then transcribed in vitro using T7 RNA polymerase according to the protocol supplied by the manufacturer (Roche). After transcription, 10 U of RQ DNase I (Promega) was added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol–chloroform and RNA was precipitated with ethanol–7.5 M ammonium acetate.

Transfection

Huh-7 cells were seeded at 1×10^5 per well of 12-well plates 24 h before transfection. 0.5 μg of replicon RNA in vitro transcribed from pLMH14 or pLMH14GHD, and 1 μg of pCnwtax, pCnm148 or pCnm319 were co-transfected into cells with Lipofectin Reagent (Invitrogen). The cells were harvested and the luciferase activity was determined at 3 h and 72 h posttransfection as described below. The luciferase level at 3 h posttransfection was used to correct transfection efficiency.

Luciferase assay

Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants was used for luciferase assays. Luciferase activity was measured using a TD-20/20 Luminometer (Promega).

Western blot analysis

Protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, transferred to Hybond-P PVDV Membrane (GE Healthcare). The blot was probed with monoclonal antibody specific for HTLV-I Tax (AS-5703, Microbix Biosystems Inc.), and signals were visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Real-time RT-PCR

RNAs were isolated from cultured cells with Trizol reagent (Invitrogen) and HCV replicon RNA was quantified by real-time RT-PCR as described previously (Zhang et al., 2005a). Briefly, 1 μg of DNase-treated total RNA was reserve transcribed and subsequently amplified with SYBR GREEN according to the protocol supplied by the manufacturer (Takara). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level in each sample was simultaneously quantified to normalize the value of HCV replicon RNA.

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