critical virion components to be introduced into the cytoplasm for subsequent processes, including uncoating, gene expression, and replication. The present study documented that the main pathway of SARS-CoV entry into host cells is dependent on clathrin. Chlorpromazine is a cationic amphiphilic agent that inhibits the formation of clathrin-coated pits (24). The use of chlorpromazine has established that a number of other viruses, including VSV (40) and influenza virus (36), use clathrin for entry into the host cell. We found that chlorpromazine inhibited the infection of HepG2 cells by SARS-CoV(HIV) more significantly than by VSV(HIV). We also confirmed the inhibitory effect of chlorpromazine on the SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells. Moreover, HepG2 cells used here are unable to form caveolae because of no expression of caveolin-1 (data not shown). These results suggest that the SARS-CoV entry is mainly mediated by clathrin-coated pits. The specificity of the chlorpromazine effect was supported by the observation that the chlorpromazine treatment had little effect on the entry of the clathrin-independent A-MLV(HIV). To verify SARS-CoV's dependence on clathrin for host cell entry, we used CHC knockdown HepG2 cells as the target for SARS-CoV infection. The treatment of HepG2 cells with CHC siRNA induced significant suppression of the SARS-CoV(HIV) infection, as well as the SARS-CoV infection. Furthermore, we found that the ACE2 is colocalized with the CHC out of the lipid raft. Hence, although we cannot exclude a minor contribution of the clathrin-independent pathway to SARS-CoV(HIV) entry, the clathrin-dependent pathway is probably the main one used by SARS-CoV for host cell entry.

A previous report demonstrated that an ACE2 mutant partially deleted of the cytoplasmic domain sustains a receptor activity for SARS-CoV infection (11). Since we found that the ACE2-mediated infection of SARS-CoV is mainly clathrin dependent, we then definitively sought to determine whether the cytoplasmic domain of ACE2 is required for the clathrin-dependent pathway of SARS-CoV infection. We prepared an ACE2 mutant, ACE2-Δtail, with the cytoplasmic domain completely deleted, and demonstrated that the SARS-CoV infection via the ACE2-Δtail is mainly clathrin dependent because chlorpromazine inhibits the ACE2-Δtail-mediated entry of SARS-CoV(HIV) into COS7 cells. These results indicate that the cytoplasmic domain of the ACE2 is not essential for the clathrin-dependent entry of SARS-CoV, which suggests that there is a possible coreceptor for the ACE2, which interacts with the AP2/clathrin complex. The replacement of the transmembrane domain of ACE2 with that of EGFR showed no effect on the susceptibility of the cells to SARS-CoV. Since the ACE2 extracellular domain alone is unable to induce the receptor activity, the extracellular domain containing the transmembrane domain is indispensable for the receptor activity for SARS-CoV but the cytoplasmic tail of ACE2 is dispensable.

SARS-CoV infection was previously shown to be suppressed partially by treatment of the cholesterol-depleting reagent, MBCD (19). We also observed lower but significant levels of MBCD-mediated suppression of the SARS-CoV(HIV) and VSV(HIV) infection compared to the A-MLV(HIV) infection. The difference in MBCD's suppressive effects between A-MLV(HIV) and the other two pseudoviruses may be explained by the possibility that the SARS-CoV(HIV) entry might be partially mediated by a clathrin-independent pathway corresponding to the lipid raft-mediated pathway. Alternatively, in a previous report, MBCD partially inhibited the clathrin-mediated endocytosis of transferrin receptor and EGFR, as well as completely inhibiting their lipid raft- and/or caveola-mediated endocytosis (14, 33), suggesting that MBCD might have some unexpected effect on the clathrin-mediated pathway for the SARS-CoV infection. To address these possibilities, we investigated the additive or synergistic effect of MBCD with CHC-siRNA on the SARS-CoV infection to HepG2 cells. MBCD induced a weak but significant additive suppression on CHC-siRNA-treated HepG2 cell infection by SARS-CoV, suggesting a minor clathrin-independent entry pathway for SARS-CoV.

The SARS-CoV S protein is cleaved into S1 and S2 proteins by an acidic protease, cathepsin L, in endosomes, which is essential for fusion between the viral envelope and the endosome vesicular membrane (1, 13, 37). We demonstrated here the translocation of ACE2 to the EEA1-positive endosomes upon SARS-CoV(HIV) infection and showed the suppressive effects of acidification inhibitors, NH_4Cl and chloroquine, on SARS-CoV(HIV) infection to HepG2 cells. Furthermore, the SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail was significantly suppressed by NH_4Cl treatment, suggesting that SARS-CoV infection via ACE2-Δtail is not different from that via the wild-type ACE2.

Based on the present study, we propose a model for SARS-CoV's internalization by target cells. SARS-CoV attaches the cell surface through an interaction between the envelope spike glycoprotein and its receptor, ACE2. Clathrin-coated pits are then formed by interactions between the ACE2/virus complex and the AP2/clathrin complex via a possible coreceptor in a non-lipid-raft portion of the plasma membrane. The ACE2/virus complex is then translocated to endosomes, where the virus is uncoated by the help of endosomal acid protease, such as cathepsin L (13, 37).

Among the *Coronaviridae*, human coronavirus 229E (HCoV-229E) utilizes aminopeptidase N (CD13) as its receptor, which is localized in the lipid rafts, and HCoV-229E infection is inhibited by the treatment of target cells with MBCD and the transfection of an siRNA specific for caveolin-1, suggesting that HCoV-229E utilizes the caveola-mediated endocytosis pathway for its entry into target cells (26). Since CD13 is a common viral receptor for other group 1 coronaviruses, such as porcine transmissible gastroenteritis virus, porcine epidemic diarrhea virus, feline infectious peritonitis virus, and canine coronavirus, the caveola-mediated endocytosis pathway seems to be conserved among them. In contrast, murine hepatitis virus, which is a group 2 coronavirus, was previously shown to utilize the lipid raft-mediated endocytosis pathway (5). Hence, SARS-CoV, which is also classified into the group 2 coronavirus, seems to be unique among the *Coronaviridae* because it utilizes the clathrin-mediated pathway for its entry into HepG2 and COS7 cells.

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REFERENCES

1. **Ashraf, H.** 2005. Cathepsin enzyme provides clue to SARS infection. Drug Discov. Today **10**:1409.
2. **Beer, C., D. S. Andersen, A. Rojek, and L. Pedersen.** 2005. Caveola-dependent endocytic entry of amphotropic murine leukemia virus. J. Virol. **79**: 10776–10787.
3. **Belleudi, F., V. Visco, M. Ceridono, L. Leone, R. Muraro, L. Frati, and M. R. Torrisi.** 2003. Ligand-induced clathrin-mediated endocytosis of the keratinocyte growth factor receptor occurs independently of either phosphorylation or recruitment of eps15. FEBS Lett. **553**:262–270.
4. **Bousarghin, L., A. Touze, P. Y. Sizaret, and P. Coursaget.** 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. J. Virol. **77**:3846–3850.
5. **Choi, K. S., H. Aizaki, and M. M. Lai.** 2005. Murine coronavirus requires lipid rafts for virus entry and cell-cell fusion but not for virus release. J. Virol. **79**:9862–9871.
6. **Damm, E. M., L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, and A. Helenius.** 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J. Cell Biol. **168**:477–488.
7. **Danthi, P., and M. Chow.** 2004. Cholesterol removal by methyl-beta-cyclodextrin inhibits poliovirus entry. J. Virol. **78**:33–41.
8. **Empig, C. J., and M. A. Goldsmith.** 2002. Association of the caveola vesicular system with cellular entry by filoviruses. J. Virol. **76**:5266–5270.
9. **Gembardt, F., A. Sterner-Kock, H. Imboden, M. Spalteholz, F. Reibitz, H. P. Schultheiss, W. E. Siems, and T. Walther.** 2005. Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents. Peptides **26**:1270–1277.
10. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
11. **Hofmann, H., M. Geier, A. Marzi, M. Krumbiegel, M. Peipp, G. H. Fey, T. Gramberg, and S. Pohlmann.** 2004. Susceptibility to SARS coronavirus S protein-driven infection correlates with expression of angiotensin converting enzyme 2 and infection can be blocked by soluble receptor. Biochem. Biophys. Res. Commun. **319**:1216–1221.
12. **Hommelgaard, A. M., K. Roepstorff, F. Vilhardt, M. L. Torgersen, K. Sandvig, and B. van Deurs.** 2005. Caveolae: stable membrane domains with a potential for internalization. Traffic **6**:720–724.
13. **Huang, I. C., B. J. Bosch, F. Li, W. Li, K. H. Lee, S. Ghiran, N. Vasilieva, T. S. Dermody, S. C. Harrison, P. R. Dormitzer, M. Farzan, P. J. Rottier, and H. Choe.** 2006. SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. J. Biol. Chem. **281**:3198–3203.
14. **Imelli, N., O. Meier, K. Boucke, S. Hemmi, and U. F. Greber.** 2004. Cholesterol is required for endocytosis and endosomal escape of adenovirus type 2. J. Virol. **78**:3089–3098.
15. **Insel, P. A., B. P. Head, R. S. Ostrom, H. H. Patel, J. S. Swaney, C. M. Tang, and D. M. Roth.** 2005. Caveolae and lipid rafts: G protein-coupled receptor signaling microdomains in cardiac myocytes. Ann. N. Y. Acad. Sci. **1047**: 166–172.
16. **Kuhn, J. H., W. Li, H. Choe, and M. Farzan.** 2004. Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus. Cell Mol. Life Sci. **61**:2738–2743.
17. **Kyuuma, M., K. Kikuchi, K. Kojima, Y. Sugawara, M. Sato, N. Mano, J. Goto, T. Takeshita, A. Yamamoto, K. Sugamura, and N. Tanaka.** 2007. AMSH, an ESCRT-III associated enzyme, deubiquitinates cargo on MVB/late endosomes. Cell Struct. Funct. **31**:159–172.
18. **Lambert, S., D. Vind-Kezunovic, S. Karvinen, and R. Gniadecki.** 2006. Ligand-independent activation of the EGFR by lipid raft disruption. J. Investig. Dermatol. **126**:954–962.
19. **Li, G. M., Y. G. Li, M. Yamate, S. M. Li, and K. Ikuta.** 2007. Lipid rafts play an important role in the early stage of severe acute respiratory syndrome-coronavirus life cycle. Microbes Infect. **9**:96–102.
20. **Li, W., M. J. Moore, N. Vasilieva, J. Sui, S. K. Wong, M. A. Berne, M. Somasundaran, J. L. Sullivan, K. Luzuriaga, T. C. Greenough, H. Choe, and M. Farzan.** 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426**:450–454.
21. **Marjomaki, V., V. Pietiainen, H. Matilainen, P. Upla, J. Ivaska, L. Nissinen, H. Reunanen, P. Huttunen, T. Hyypia, and J. Heino.** 2002. Internalization of echovirus 1 in caveolae. J. Virol. **76**:1856–1865.
22. **Marsh, M., and A. Helenius.** 2006. Virus entry: open sesame. Cell **124**:729–740.
23. **Mellman, I.** 1996. Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. **12**:575–625.
24. **Nawa, M., T. Takasaki, K. Yamada, I. Kurane, and T. Akatsuka.** 2003. Interference in Japanese encephalitis virus infection of Vero cells by a cationic amphiphilic drug, chlorpromazine. J. Gen. Virol. **84**:1737–1741.
25. **Nie, Y., P. Wang, X. Shi, G. Wang, J. Chen, A. Zheng, W. Wang, Z. Wang, X. Qu, M. Luo, L. Tan, X. Song, X. Yin, M. Ding, and H. Deng.** 2004. Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression. Biochem. Biophys. Res. Commun. **321**: 994–1000.
26. **Nomura, R., A. Kiyota, E. Suzaki, K. Kataoka, Y. Ohe, K. Miyamoto, T. Senda, and T. Fujimoto.** 2004. Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. J. Virol. **78**:8701–8708.
27. **Pearse, B. M., C. J. Smith, and D. J. Owen.** 2000. Clathrin coat construction in endocytosis. Curr. Opin. Struct. Biol. **10**:220–228.
28. **Pelkmans, L.** 2005. Secrets of caveolae- and lipid raft-mediated endocytosis revealed by mammalian viruses. Biochim. Biophys. Acta **1746**:295–304.
29. **Pelkmans, L., and A. Helenius.** 2003. Insider information: what viruses tell us about endocytosis. Curr. Opin. Cell Biol. **15**:414–422.
30. **Pietiainen, V. M., V. Marjomaki, J. Heino, and T. Hyypia.** 2005. Viral entry, lipid rafts and caveosomes. Ann. Med. **37**:394–403.
31. **Prabakaran, P., X. Xiao, and D. S. Dimitrov.** 2004. A model of the ACE2 structure and function as a SARS-CoV receptor. Biochem. Biophys. Res. Commun. **314**:235–241.
32. **Riemann, D., G. H. Hansen, L. Niels-Christiansen, E. Thorsen, L. Immerdal, A. N. Santos, A. Kehlen, J. Langner, and E. M. Danielsen.** 2001. Caveolae/lipid rafts in fibroblast-like synoviocytes: ectopeptidase-rich membrane microdomains. Biochem. J. **354**:47–55.
33. **Rodal, S. K., G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, and K. Sandvig.** 1999. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol. Biol. Cell **10**:961–974.
34. **Sha, Y., Y. Wu, Z. Cao, X. Xu, W. Wu, D. Jiang, X. Mao, H. Liu, Y. Zhu, R. Gong, and W. Li.** 2006. A convenient cell fusion assay for the study of SARS-CoV entry and inhibition. IUBMB Life. **58**:480–486.
35. **Sieczkarski, S. B., and G. R. Whittaker.** 2005. Characterization of the host cell entry of filamentous influenza virus. Arch. Virol. **150**:1783–1796.
36. **Sieczkarski, S. B., and G. R. Whittaker.** 2003. Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses. Traffic **4**:333–343.
37. **Simmons, G., D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond, and P. Bates.** 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. USA **102**:11876–11881.
38. **Sorkin, A.** 2004. Cargo recognition during clathrin-mediated endocytosis: a team effort. Curr. Opin. Cell Biol. **16**:392–399.
39. **Stang, E., F. D. Blystad, M. Kazazic, V. Bertelsen, T. Brodahl, C. Raiborg, H. Stenmark, and I. H. Madshus.** 2004. Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. Mol. Biol. Cell **15**:3591–3604.
40. **Sun, X., V. K. Yau, B. J. Briggs, and G. R. Whittaker.** 2005. Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology **338**:53–60.
41. **Yang, Z. Y., Y. Huang, L. Ganesh, K. Leung, W. P. Kong, O. Schwartz, K. Subbarao, and G. J. Nabel.** 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. J. Virol. **78**:5642–5650.
42. **Yao, D., M. Ehrlich, Y. I. Henis, and E. B. Leof.** 2002. Transforming growth factor-beta receptors interact with AP2 by direct binding to beta2 subunit. Mol. Biol. Cell **13**:4001–4012.
43. **Yu, F., M. Q. Le, S. Inoue, H. T. Thai, F. Hasebe, M. Del Carmen Parquet, and K. Morita.** 2005. Evaluation of inapparent nosocomial severe acute respiratory syndrome coronavirus infection in Vietnam by use of highly specific recombinant truncated nucleocapsid protein-based enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **12**:848–854.
44. **Ziebuhr, J.** 2004. Molecular biology of severe acute respiratory syndrome coronavirus. Curr. Opin. Microbiol. **7**:412–419.

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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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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.

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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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-

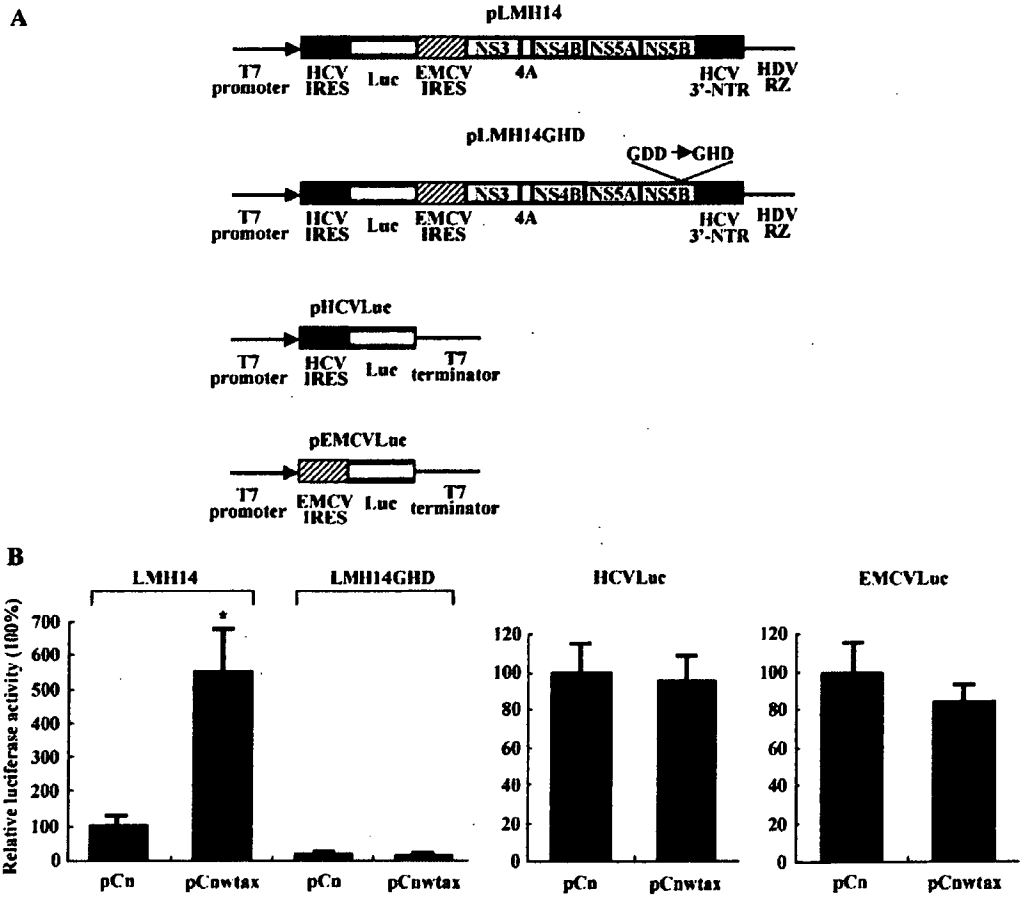

Fig. 1. Activation of HCV replication by HTLV-I Tax. (A) Schematic diagrams of the HCV replicon construct pLMH14, the replication-deficient replicon pLMH14GHD, as well as two control reporter vectors pHCVLuc and pEMCVLuc. pLMH14 contains T7 promoter, 5′ nontranslated region (NTR), the first 36 nucleotides 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.

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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-1 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

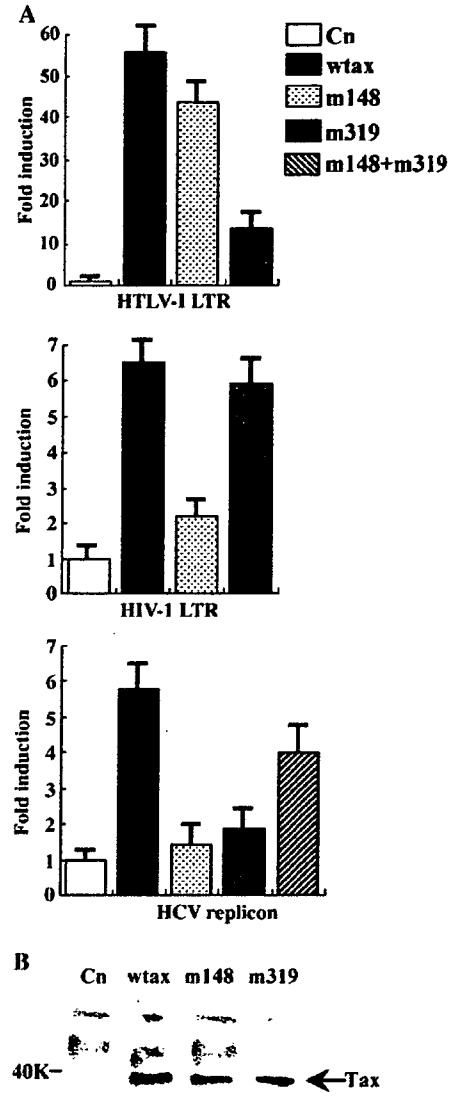

Fig. 2. Effect of Tax or its mutants on HCV replication. (A) Huh-7 cells were transfected with pHTLVLTR-Luc (upper), pHIVLTR-Luc (middle) or the replicon RNA in vitro transcribed from pLMH14 (lower), along with pCn, pCnwtax, pCnm148 or pCnm319. Relative luciferase activities were determined as described 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.

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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

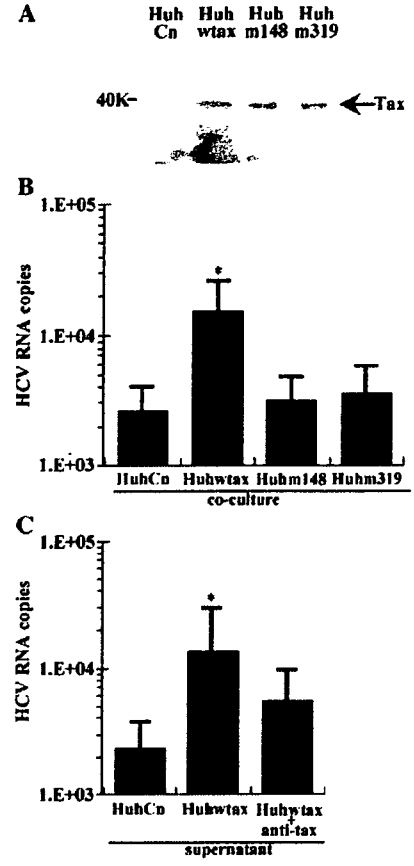

Fig. 3. Paracrine effect of HTLV-I Tax on HCV replication. (A) Similar level of Tax expression in cell lines stably expressing wild- or mutant-type Tax. Huh-7 cells were transfected with pCn, pCnwtax, pCnm148 or pCnm319 followed by G418 selection, and Tax expression was detected by Western blot analysis. 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

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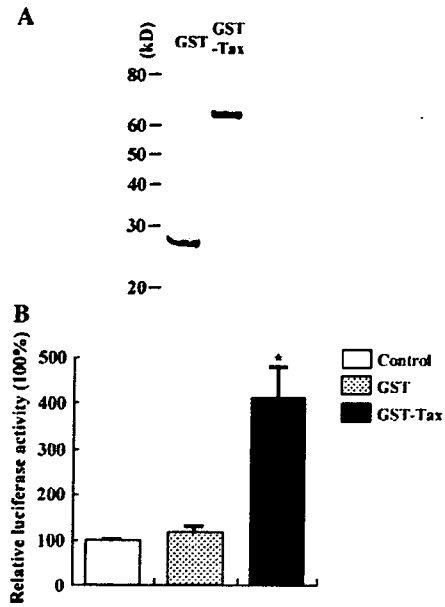

Fig. 4. Up-regulation of HCV replication by recombinant Tax protein. (A) SDS-PAGE analysis of purified GST and GST-Tax proteins. The gel was stained with Coomassie blue. (B) Huh-7 cells transfected with replicon RNA was cultured in the absence or presence of 5 nM GST or GST-Tax for 72 h, and the relative 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

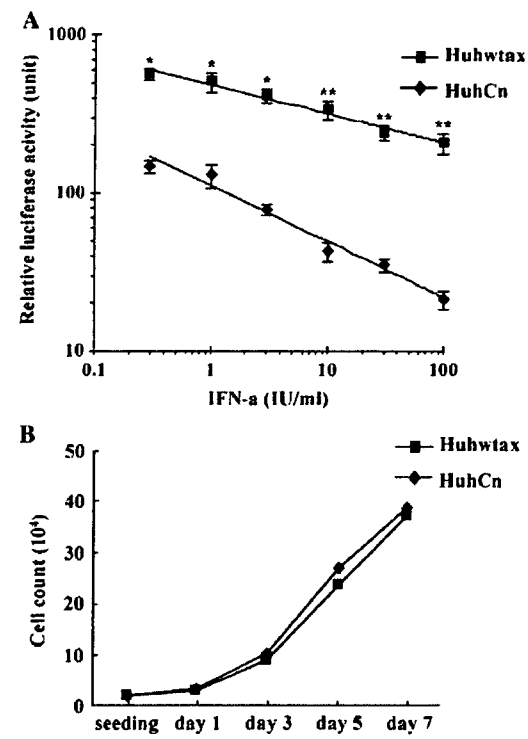

Fig. 5. (A) Modulation of interferon (IFN)-α responsiveness of HCV replication by HTLV-I Tax. HuhCn or Huhwtax cells were transfected with the replicon RNA in vitro transcribed from pI.MH14, harvested and divided into several aliquots at 3 h posttransfection, which were further incubated for 3 days in 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.

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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

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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.

References

Alefantis, T., Mostoller, K., Jain, P., Harhaj, E., Grant, C., Wigdahl, B., 2005. Secretion of the human T cell leukemia virus type I transactivator protein Tax. J. Biol. Chem. 280 (17), 17353–17362.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V., Vogelstein, B., 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249 (4971), 912–915.

Boschi-Pinto, C., Stuver, S., Okayama, A., Trichopoulos, D., Orav, E.J., Tsubouchi, H., Mueller, N., 2000. A follow-up study of morbidity and mortality associated with hepatitis C virus infection and its interaction with human T lymphotropic virus type I in Miyazaki, Japan. J. Infect. Dis. 181 (1), 35–41.

Brand, S.R., Kobayashi, R., Mathews, M.B., 1997. The Tat protein of human immunodeficiency virus type 1 is a substrate and inhibitor of the interferon-induced, virally activated protein kinase, PKR. J. Biol. Chem. 272 (13), 8388–8395.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis C virus genome. Science 244 (4902), 359–362.

Cowan, E.P., Alexander, R.K., Daniel, S., Kashanchi, F., Brady, J.N., 1997. Induction of tumor necrosis factor alpha in human neuronal cells by extracellular human T-cell lymphotropic virus type 1 Tax1. J. Virol. 71 (9), 6982–6989.

De Rossi, A., Saggioro, D., Calabro, M.L., Cenzato, R., Chieco-Bianchi, L., 1991. Reciprocal activation of human T-lymphotropic viruses in HTLV-I-transformed cells superinfected with HIV-1. J. Acquir. Immune Defic. Syndr. 4 (4), 380–385.

Gessain, A., Barin, F., Vernant, J.C., Gout, O., Maurs, L., Calender, A., de The, G., 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. Lancet 2 (8452), 407–410.

Kishihara, Y., Furusyo, N., Kashiwagi, K., Mitsutake, A., Kashiwagi, S., Hayashi, J., 2001. Human T lymphotropic virus type I infection influences hepatitis C virus clearance. J. Infect. Dis. 184 (9), 1114–1119.

Kishine, H., Sugiyama, K., Hijikata, M., Kato, N., Takahashi, H., Noshi, T., Nio, Y., Hosaka, M., Miyanari, Y., Shimotohno, K., 2002. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. BBRC 293 (3), 993–999.

Look, D.C., Roswit, W.T., Frick, A.G., Gris-Alevy, Y., Dickhaus, D.M., Walter, M.J., Holtzman, M.J., 1998. Direct suppression of stat1 function during adenoviral infection. Immunity 9 (6), 871–880.

Major, M.E., Feinstone, S.M., 1997. The molecular virology of hepatitis C. Hepatology 25 (6), 1527–1538.

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Marriott, S.J., Trinh, D., Brady, J.N., 1992. Activation of interleukin-2 receptor alpha expression by extracellular HTLV-I Tax1 protein: a potential role in HTLV-I pathogenesis. Oncogene 7 (9), 1749–1755.

Moch, H., Lang, D., Stamminger, T., 1992. Strong trans activation of the human cytomegalovirus major immediate-early enhancer by $p40^{tax}$ of human T-cell leukemia virus type I via two repetitive Tax-responsive sequence elements. J. Virol. 66 (12), 7346–7354.

Mori, N., Fujii, M., Cheng, G., Ikeda, S., Yamasaki, Y., Yamada, Y., Tomonaga, M., Yamamoto, N., 2001. Human T-cell leukemia virus type I tax protein induces the expression of anti-apoptotic gene Bcl-xL in human T-cells through nuclear factor-kappaB and c-AMP responsive element binding protein pathways. Virus Genes 22 (3), 279–287.

Moriuchi, M., Moriuchi, H., 2006. Induction of lactoferrin gene expression in myeloid or mammary gland cells by human T-cell leukemia virus type 1 (HTLV-1) Tax: implication for milk-borne transmission of HTLV-1. J. Virol. 80 (14), 7118–7126.

Moriuchi, H., Moriuchi, M., Fauci, A.S., 1998. Factors secreted by human T lymphotropic virus type 1 (HTLV-I)-infected cells can enhances or inhibit replication of HIV-1 in HTLV-I-uninfected cells: implication for in vivo coinfection with HTLV-I and HIV-1. J. Exp. Med. 187 (10), 1689–1697.

Murata, T., Ohshima, T., Yamaji, M., Hosaka, M., Miyanari, Y., Hijikata, M., Shimotohno, K., 2005. Suppression of hepatitis C virus replicon by TGF-β. Virology 331 (2), 407–417.

Nakamura, M., Niki, M., Nagata, K., Ohtani, K., Saito, S., Hinuma, Y., Sugamura, K., 1989. Cell line-dependent response of the enhancer element of simian virus 40 to transactivator p40tax encoded by human T-cell leukemia virus type I. J. Biol. Chem. 264 (34), 20189–20192.

Okada, Y., Sawa, H., Tanaka, S., Takada, A., Suzuki, S., Hasegawa, H., Umemura, T., Fujisawa, J., Tanaka, Y., Hall, W.W., Nagashima, K., 2000. Transcriptional activation of JC virus by human T-lymphotropic virus type I Tax protein in human neuronal cell lines. J. Biol. Chem. 275 (22), 17016–17023.

Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., 1986. HTLV-I associated myelopathy, a new clinical entity. Lancet 1 (8488), 1031–1032.

Pietschmann, T., Lohmann, V., Rutter, G., Kurpanek, K., Bartenschlager, R., 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. 75 (3), 1252–1264.

Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B.A., Lemon, S.M., 2004. Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. J. Virol. 78 (3), 1513–1524.

Sharrocks, A.D., 1994. A T7 expression vector for producing N- and C-terminal fusion proteins with glutathione S-transferase. Gene 138 (1-2), 105–108.

Siekevitz, M., Josephs, S.F., Dukovich, M., Peffer, N., Wong-Staal, F., Greene, W.C., 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science 238 (4833), 1575–1578.

Szabo, J., Bacsi, A., Andirko, I., Kiss, J., Nemes, J., Toth, F.D., 1998. Reciprocal interactions between human cytomegalovirus and human T cell leukemia-lymphoma virus type I in monocyte-derived macrophages cultured in vitro. AIDS Res. Hum. Retrovir. 14 (8), 699–709.

Szabo, J., Bacsi, A., Beck, Z., Kiss, J., Andirko, I., Toth, F.D., 1999. Role of interleukin-8 and transforming growth factor-beta1 in enhancement of human cytomegalovirus replication by human T cell leukemia-lymphoma virus type I in macrophages coinfected with both viruses. J. Interferon Cytokine Res. 19 (2), 209–217.

Szymocha, R., Akaoka, H., Dutuit, M., Malcus, C., Didier-Bazes, M., Belin, M.F., Giraudon, P., 2000. Human T-cell lymphotropic virus type 1-infected T lymphocytes impair catabolism and uptake of glutamate by astrocytes via Tax-1 and tumor necrosis factor alpha. J. Virol. 74 (14), 6433–6441.

Toth, F.D., Aboagye-Mathiesen, G., Szabo, J., Liu, X., Mosborg-Petersen, P., Kiss, J., Hager, H., Zdravkovic, M., Andirko, I., Aranyosi, J., 1995. Bidirectional enhancing activities between human T cell leukemia Lymphoma virus type I and human cytomegalovirus in human term syncytiotrophoblast cells cultured in vitro. AIDS Res. Hum. Retrovir. 11 (12), 1495–1507.

Yamaoka, S., Inoue, H., Sakurai, M., Sugiyama, T., Hazama, M., Yamada, T., Hatanaka, M., 1996. Constitutive activation of NF-kB is essential for transformation of rat fibroblasts by the human T-cell leukemia virus type I Tax protein. EMBO J. 15 (4), 873–887.

Yoshida, M., Seiki, M., Yamaguchi, K., Takatsuki, K., 1984. Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease. Proc. Natl. Acad. Sci. U.S.A. 81 (19), 2534–2537.

Zhang, J., Yamada, O., Sakamoto, T., Yoshida, H., Iwai, T., Matsushita, Y., Shimamura, H., Araki, H., Shimotohno, K., 2004. Down-regulation of viral replication by adenoviral-mediated expression of siRNA against cellular cofactors for hepatitis C virus. Virology 320 (1), 135–143.

Zhang, J., Yamada, O., Sakamoto, T., Yoshida, H., Araki, H., Shimotohno, K., 2005a. Exploiting cis-acting replication elements to direct hepatitis C virus-dependent transgene expression. J. Virol. 79 (10), 5923–5932.

Zhang, J., Yamada, O., Sakamoto, T., Yoshida, H., Araki, H., Murata, T., Shimotohno, K., 2005b. Inhibition of hepatitis C virus replication by pol III-directed overexpression of RNA decoys corresponding to stem-loop structures in the NS5B coding region. Virology 342 (2), 276–285.

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Images in *Thorax*

Secondary bronchiolitis obliterans organising pneumonia in a patient with carbamazepine-induced hypogammaglobulinemia

A 49-year-old woman had been treated with carbamazepine for 2 years because of epilepsy. She was referred to us for progressive exertional dyspnea and prolonged productive cough. Chest computed tomography (CT) scan showed bilateral infiltrates including ground glass opacities and consolidations predominantly in the lower lung fields. Her laboratory findings showed severe hypogammaglobulinemia, that is, immunoglobulin (Ig) G 418 mg/dl (normal, 748–1694 mg/dl), Ig A 20 mg/dl (91–391 mg/dl) and Ig M 51 mg/dl (33–254 mg/dl). Carbamazepine and other suspected antibiotics were all negative for drug-induced lymphocyte stimulation tests. Histological examination by trans-bronchial lung biopsy showed intraluminal fibrosis of distal airspaces with foamy alveolar macrophages, suggesting bronchiolitis obliterans organising pneumonia (BOOP). After the cessation of carbamazepine, all abnormalities in gammaglobulins and roentgenogram findings gradually improved without any medication. This good clinical course also considerably supports the diagnosis of BOOP.

BOOP may result from diverse causes such as drugs, acute respiratory infections and radiation treatment, or appear idiopathically.[1 2] Here, we show a case of secondary BOOP, which was associated with repeated respiratory infections caused by carbamazepine-induced hypogammaglobulinemia. Although the exact mechanisms of carbamazepine-induced hypogammaglobulinemia are unknown, they can be classified into three groups, that is, an absence of B cells,[3] an extensive impairment of the synthesis of Igs in B cells[3] and a disorder of the class-switch of Igs in B cells.[4] Our case described above would belong to the second group. Generally, drug-induced BOOP often develops within several weeks or less. However, our report indicates that even in the case of several years after use, anticonvulsants such as carbamazepine may have some adverse effects on the immune system and cause frequent airway infections, resulting in the development of secondary BOOP.

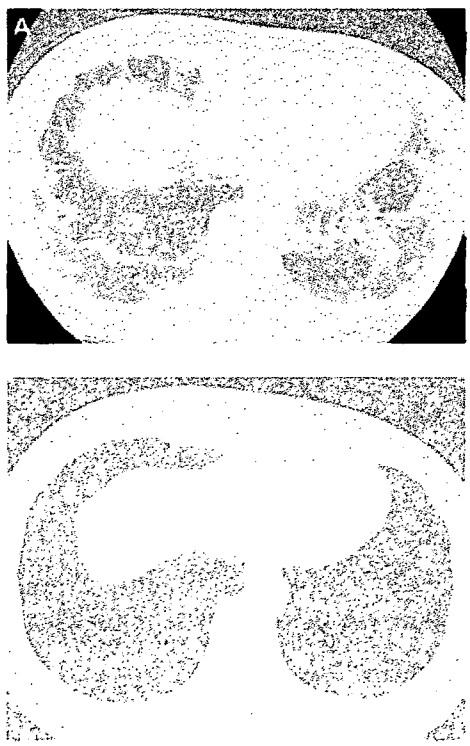

Figure 1 (A) Chest CT scans on admission. Bilateral infiltrates including ground glass opacities and consolidations are seen predominantly in lower lung fields. (B) Chest CT scans seven months after the cessation of carbamazepine showing marked improvement. The serum levels of Ig G, Ig A and Ig M are also increased to 1328, 69 and 355 mg/dl, respectively.

Learning points

- A drug-induced hypogammaglobulinemia after long term use of carbamazepine is very rare.
- A hypogammaglobulinemia should be considered as one of the causes of secondary bronchiolitis obliterans organising pneumonia with repeated airway infections.

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REFERENCES

1 **Cordier J-F.** Organising pneumonia. *Thorax* 2000;**55**:318–28.
2 **Cazzato S,** Zompatori M, Baruzzi G, *et al.* Bronchiolitis obliterans-organizing pneumonia: an Italian experience. *Respir Med* 2000;**94**:702–8.
3 **Castro AP,** Redmershi MG, Pastorino AC, *et al.* Secondary hypogammaglobulinemia after use of carbamazepine: case report and review. *Rev Hosp Clin Fac Med Sao Paulo* 2001;**56**(6):189–92.
4 **Go T.** Carbamazepine-induced IgG1 and IgG2 deficiency associated with B cell maturation defect. *Seizure* 2004;**13**:187–90.

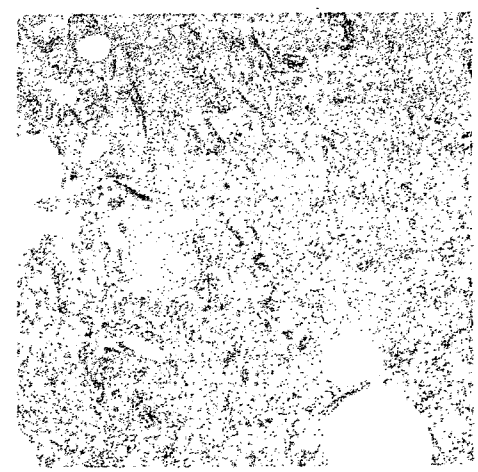

Figure 2 Elastica-Masson staining of specimens from TBLB. Immature fibroblastic foci and foamy alveolar macrophages are obstructing the alveolar ducts and adjacent alveoli. These features are consistent with BOOP.

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Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung

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Abstract

The involvement of inflammation in the pathogenesis of chronic obstructive pulmonary disease (COPD) has been investigated using samples from relatively central airways such as airway biopsies, but there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process. To determine the molecules that relate to the mechanisms underlying the pathogenesis of COPD, we evaluated the mRNA expression of inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from 33 COPD and non-COPD subjects who were undergoing lung resection for lung cancer using an RT-PCR technique.

Among the 42 studied candidate genes, the expressions of mRNA for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) were significantly decreased in COPD lung tissues compared with those in non-COPD tissues, and most of these decreases were significantly correlated with the degree of airflow limitation. On the other hand, the expressions of mRNA for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) were significantly increased in COPD lungs. Most of these changes were also associated with cigarette smoking.

These data suggest that an impairment of protective mechanisms against oxidants and xenobiotics, in addition to the upregulation of CXC- and CC-chemokines, may be associated with cigarette smoking and involved in the inflammatory process of COPD.

Keywords: Catalase; Glutathion *S*-transferase P1; Glutathion *S*-transferase M1; Microsomal epoxide hydrolase; Tissue inhibitor of metalloproteinase 2; mRNA; RT-PCR

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a disease characterized by airway inflammation and progressive airflow limitation that is not fully reversible. The morbidity and mortality of the disease have increased in recent years and it is a serious public health problem in many countries throughout the world [1]. Abnormal inflammatory responses of the lungs against noxious gases and particles, such as cigarette smoke, are thought to cause small airway disease, namely obstructive bronchiolitis, and parenchymal destruction, leading to the pathophysiologic changes of COPD such as airflow limitation [2]. Although the precise mechanisms of these processes have not been fully clarified, several mechanisms have been suggested to contribute to the disease process.

The cellular inflammatory response in COPD is characterized by increases in neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. Inflammatory mediators from these cells and epithelial cells contribute to interactions among these cells and cause the

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pathophysiological changes of COPD including mucus hypersecretion, fibrosis, and parenchymal destruction [4]. Oxidative stress, which may result from excessive oxidants and/or impaired antioxidant activities, is thought to be one of the major causes of inflammation and injury in diseased lungs. Oxidative stress arises during the inflammatory process in the lung and also from the environment. Both endogenous reactive oxygen species released from inflammatory cells such as neutrophils and macrophages and oxidant compounds in cigarette smoke or air pollution cause injuries to lung tissues [5]. These harmful molecules are eliminated by antioxidant enzyme activities in normal lungs [6]. In COPD lungs, these protective mechanisms seem to be impaired. Another mechanism in the COPD pathogenesis is an imbalance of proteinase and antiproteinase. α_1-antitrypsin has been shown to be involved in the structural changes in COPD, and a number of proteinases and antiproteinases have been reported to play roles in the inflammatory process in COPD [7].

The involvement of inflammation, oxidative stress and a proteinase/antiproteinase imbalance in the pathogenesis of COPD has been investigated using samples from COPD subjects. Many studies employing samples from relatively central airways such as airway biopsies and induced sputum have revealed an alteration in the formation of inflammatory mediators, oxidant, antioxidant, proteinase and antiproteinase in COPD airways. However, there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process.

In this study, we evaluated the expressions of 42 genes for inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from COPD and non-COPD subjects. We found decreased mRNA expressions for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) and increased expressions for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) in the COPD lung. Most of these changes were associated with the degree of airflow limitation and cigarette smoking.

2. Materials and methods

2.1. Subjects

Thirty-three patients with or without COPD who were undergoing lung resection for lung cancer took part in the study after giving written informed consent. This study was approved by the Tohoku University Committee on Clinical Investigations and by the Ethics Review Board of Miyagi Prefectural Cancer Center. According to the presence or absence of COPD and a history of smoking, the subjects were divided into three groups: 10 non-COPD subjects who never smoked, 9 non-COPD smokers and 14 COPD subjects. All patients with COPD satisfied the Global Initiative for Chronic Obstructive Lung Disease guidelines [2] and were diagnosed as having pulmonary emphysema through computed tomography. The smokers were divided into current smokers and ex-smokers, defined as those who had quit smoking for at least 3 months prior to the study. The clinical characteristics of the study subjects are shown in Table 1. All patients were stable and had no respiratory tract infection during the month preceding the study. Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), maximum flow rate at 50% of vital capacity divided by measured body height (V_{50}/HT) and maximum flow rate at 25% of vital capacity divided by measured body height (V_{25}/HT) were measured using a dry rolling-seal spirometer (FUDAC-70; Fukuda Denshi Co., Ltd., Tokyo, Japan) in the week before surgery.

2.2. Lung tissue collection

Peripheral lung tissue was obtained from the subpleural parenchyma of the lobe resected at surgery, avoiding areas involved by tumor. The tissue specimen (size 5 × 5 × 10 mm) was immediately immersed in Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and kept at −80 °C until RT-PCR analysis.

2.3. Isolation of total RNA and real-time quantitative PCR

Total RNA from each sample was extracted in guanidine isothiocyanate and phenol (Isogen) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed with random hexamer and MultiScribe Reverse Transcriptase using TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA). cDNA samples corresponding to 8 ng of total RNA were measured by real-time quantitative PCR using Applied Biosystems prism 7900HT Sequence Detection System according to the manufacturer's instructions. The primers and probes were

Table 1
Characteristics of study subjects

	Non-COPD		COPD
	Never smoked	Smokers	
Number	10	9	14
Male/Female	2/8	5/4	12/2
Age (year)	66.6±2.5	57.1±5.3	72.9±1.5††
Smoking status (ex/current)	—	5/4	11/3
Smoking history (pack-year)	0	21.6±6.7**	51.6±6.0**
%FVC (%)	122.3±8.3	107.3±3.0	89.6±4.5**
%FEV_1 (%)	135.6±9.7	111.7±3.2	78.5±6.7**
FEV_1/FVC% (%)	79.8±1.4	77.2±1.0	58.6±3.0**

The data are expressed as mean±SEM.
**$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney *U*-test).
††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney *U*-test).

obtained from TaqMan Gene Expression Assays or Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA) or designed for each of the genes according to the Primer Express 2.0 program provided by Applied Biosystems (Table 2). Reporter dyes and quencher dyes for all designed probes were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), respectively.

The amount of target message in each sample was estimated from a threshold cycle number (CT), which is inversely correlated with the number in its initial mRNA level and used to determine gene expression. The CT-values were generated by the ABI PRISM 7900HT SDS software version 2.0. The CT value of each gene was normalized using the formula $\Delta CT = (CT \text{ of each gene}) - (CT \text{ of } \beta\text{-actin})$. The expression levels of each gene per β-actin were calculated according to the formula $2^{-(\Delta CT)}$.

2.4. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was carried out using nonparametric analysis of variance (Kruskal–Wallis test) to evaluate variance among the three groups. If a significant variance was found, an unpaired two-group test (Mann–Whitney *U*-test) was used to determine significant differences between individual groups. For analysis within group correlations, Spearman's rank correlation test was used. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. mRNA expressions of the 42 studied candidate genes

Table 3 shows all the data of the mRNA amounts observed in this study. We found some significant differences between COPD and non-COPD subjects. The expressions of mRNA for catalase, GSTP1, mEPHX, GSTM1 and TIMP2 were significantly decreased in COPD lung tissues compared with those in non-COPD tissues. On the other hand, the mRNA expressions for IL-1β, IL-8, Gro-α and MCP-1 were significantly increased in COPD compared with non-COPD. None of the other studied genes showed significant changes in mRNA expression in COPD versus non-COPD subjects.

3.2. Decreased expression of enzymes protective against oxidants and xenobiotics

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly decreased in COPD lung tissues compared with those in non-COPD tissues (Fig. 1). The expression of mRNA for catalase was significantly decreased in lung tissues from COPD subjects (0.165 ± 0.013) compared with those from non-COPD smokers (0.233 ± 0.026, $P = 0.023$) and those who never smoked (0.251 ± 0.025, $P = 0.003$). The GSTP1 mRNA expression was significantly decreased in lung tissues from COPD subjects (0.449 ± 0.027) compared with those from non-COPD smokers (0.554 ± 0.028, $P = 0.023$) and those who never smoked (0.647 ± 0.069, $P = 0.019$). The mEPHX mRNA expression was significantly decreased in lung tissues from COPD subjects (0.153 ± 0.014) compared with those from non-COPD smokers (0.223 ± 0.014, $P = 0.003$) and those who never smoked (0.228 ± 0.028, $P = 0.022$). The expression of mRNA for GSTM1 was significantly increased in lung tissues from non-COPD smokers (0.229 ± 0.044) compared with those from non-COPD subjects who never smoked (0.136 ± 0.033, $P = 0.034$) and those from COPD subjects (0.126 ± 0.019, $P = 0.014$) (Fig. 2).

3.3. Decreased expression of antiproteinase

The expression of mRNA for TIMP2 was significantly increased in lung tissues from non-COPD smokers (1.135 ± 0.101) compared with those from non-COPD subjects who never smoked (0.873 ± 0.166, $P = 0.014$) and those from COPD subjects (0.870 ± 0.110, $P = 0.038$) (Fig. 2).

3.4. Increased expression of inflammatory cytokines and chemokines

The expressions of mRNA for IL-1β (0.203 ± 0.058 vs. 0.051 ± 0.021, $P = 0.004$), IL-8 (0.618 ± 0.188 vs. 0.071 ± 0.036, $P = 0.003$), Gro-α (0.015 ± 0.04 vs. 0.002 ± 0.001, $P = 0.005$) and MCP-1 (1.300 ± 0.316 vs. 0.286 ± 0.120, $P = 0.002$) were significantly increased in lungs from COPD subjects compared with those from subjects who never smoked. The differences in the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 between in COPD subjects and in non-COPD smokers were not statistically significant (Fig. 3).

3.5. Correlations between gene expressions and pulmonary function and cigarette smoking habit

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly correlated with the parameters of pulmonary function: % of predicted values of forced vital capacity (%FVC), % of predicted values of forced expiratory volume in one second (%FEV_1), FEV_1/FVC%, V_{50}/HT and V_{25}/HT (Fig. 4 and Table 4). The mRNA expressions for these enzymes were negatively correlated with cigarette smoking history assessed as pack-year (Table 4).

In contrast, the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 were negatively correlated with V_{50}/HT and were associated with cigarette smoking history (Table 4). The expressions of IL-8 and Gro-α were also negatively correlated with FEV_1/FVC% (Table 4).

When analyzed in two groups of non-COPD smokers and COPD subjects, the expression of mRNA for TIMP2

Table 2
Real-time quantitative PCR primers and probes

Gene	Accession no.	Sequence or assay ID	
XDH	NM_000379	Hs00166010_m1	
HMOX1	NM_002133	Hs00157965_m1	
GPX3	genbank:NM_002084NM_002084	TCCCTGCAACCAATTTGGA	Forward primer: 593–613
		CCATTGACATCCCCTTTCTCA	Reverse Primer: 723–703
		TCCTTCCTACCCTCAAGTATGTCCGACCA	TaqMan probe: 642–670
GSTM1	NM_000561	AGGACTTCATCTCCCGCTTTG	Forward primer: 599–619
		CCATCTTTGAGAACACAGGTCTTG	Reverse Primer: 691–668
		TCTGCCTACATGAAGTCCAGCCGCTT	TaqMan probe: 637–662
GSTP1	NM_000852	CCTCCGCTGCAAATACATCTC	Forward primer: 326–346
		CAGTGCCTTCACATAGTCATCCTT	Reverse Primer: 398–375
		CTCATCTACACCAACTATGAGGCGGG	TaqMan probe: 348–373
GSTT1	NM_000853	CATAAGGTGATGTTCCCTGTGTTC	Forward primer: 346–369
		GGAGATGTGAGGACCAGTAAGGA	Reverse Primer: 492–470
		TCGAGGACAAGTTCCTCCAGAACAAGGC	TaqMan probe: 440–467
SOD1	NM_000454	AAAACACGGTGGGCCAAA	Forward primer: 210–227
		ACATCGGCCACACCATCTTT	Reverse Primer: 293–274
		CCCAAGTCTCCAACATGCCTCTCTTCA	TaqMan probe: 257–231
SOD2	NM_000636	Hs00167309_m1	
SOD3	NM_003102	Hs00162090_m1	
Catalase	NM_001752	Hs00156308_m1	
mEPHX	NM_000120	Hs00164458_m1	
MMP1	NM_002421	Hs00233958_m1	
MMP2	NM_004530	Hs00234422_m1	
MMP9	NM_004994	Hs00234579_m1	
MMP12	NM_002426	Hs00159178_m1	
ELA2	NM_001972	Hs00357734_m1	
TIMP1	NM_003254	Hs00171558_m1	
TIMP2	NM_003255	Hs00234278_m1	
SERPINA1	NM_000295	Hs00165475_m1	
SLPI	NM_003064	Hs00268206_m1	
IL-13	NM_002188	H327046T	
TNF-α	NM_000594	H327055T	
TNF-α R	NM_001065	GCTTCAGAAAACCACCTCAGACA	Forward primer: 552–574
		ATGCCGGTACTGGTTCTTCCT	Reverse Primer: 683–663
		TCAGCTGCTCCAAATGCCGAAAGG	TaqMan probe: 580–603
IL-1β	NM_000576	4327035T	
IFN-α	NM_000619	4327052T	
IL-17	NM_002190	4327048T	
GM-CSF	NM_000758	4327057T	
IL-10	NM_000572	4327043T	
Gro-α	NM_001511	TTCTGAGGAGCCTGCAACATG	Forward primer: 754–774
		TCCCCTGCCTTCACAATGAT	Reverse Primer: 842–823
		CGGATCCAAGCAAATGGCCAATGA	TaqMan probe: 798–821
IL-8	NM_000584	CGGAAGGAACCATCTCACTGT	Forward primer: 73–93
		ATCAGGAAGGCTGCCAAGAGA	Reverse Primer: 145–125
		GTAAACATGACTTCCAAGCTGGCCGTG	TaqMan probe: 96–122
BLT1	NM_000752	CATCTGGGTGTTGTCCTTTCTG	Forward primer: 2137–2158
		GACAGCCTCGAAGATTAGATGGA	Reverse Primer: 2278–2256
		TGCCCTGGAAAACGAACATGAGCC	TaqMan probe: 2193–2216
BLT2	NM_019839	ACCTTCTCATCGGGCATCAC	Forward primer: 1624–1643
		GAAGTCTTCCAGCTCAGCAGTGT	Reverse Primer: 1767–1745
		CCACCTGTAGGCCCAGAAGGATGT	TaqMan probe: 1692–1715
VEGF	NM_003376	Hs00173626_m1	
MUC5AC	AJ298317	TACTCCACAGACTGCACCAACTG	Forward primer: 1263–1285
		CGTGTATTGCTTCCCGTCAA	Reverse Primer: 1391–1372
		TGTGCTTGGAGGTGCCCACTTCTCAA	TaqMan probe: 1343–1368
MUC5B	Z72496	ACCGACCACAGAGCTGGAGA	Forward primer: 863–882
		ATGTCAGTCCTTCTGAGAGGGTG	Reverse Primer: 1005–983
		TTCTCAACGCCGCAGCCTACGAGT	TaqMan probe: 912–935
MUC8	U14383	CGGCCATCATTCCTTTTTACTG	Forward primer: 1226–1247
		AACCCACATGACCATCAACTGA	Reverse Primer: 1315–1294
		CTGTGTGAATCCACCGCTAGAAACCCA	TaqMan probe: 1263–1289
CTSL	NM_145918	Hs00377632_m1	

Table 2 (*continued*)

Gene	Accession no.	Sequence or assay ID
MCP-1	NM_002982	4329524T
TGF-β	NM_000660	4327054T
EGF	NM_001963	Hs00153181_m1
IL-4	NM_000589	4327038T
IL-5	NM_000879	4327039T

Numbers refer to oligonucleotide position contained within the published cDNA.

was significantly correlated with V_{50}/HT ($r_s = 0.469$, $P = 0.028$), but not with other parameters including %FEV_1 ($r_s = 0.376$, $P = 0.077$) nor cigarette smoking history ($r_s = -0.342$, $P = 0.106$). The expression of mRNA for GSTM1 had no significant relationship with parameters for airflow limitation (data not shown) and cigarette smoking history ($r_s = -0.393$, $P = 0.065$).

In smokers with and without COPD, there was no association between the mRNA expressions for these molecules and duration since smoking cessation (data not shown).

4. Discussion

In this study, we observed decreased expressions of mRNA for catalase, GSTP1, GSTM1, mEPHX and TIMP2 and increased expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 in peripheral lungs from patients with COPD. Most of these changes in mRNA expressions were associated with the degree of airflow limitation and with the cigarette smoking habit.

Although the precise mechanisms of the pathogenesis of COPD have not been fully elucidated, it is thought that the inflammatory responses of the lungs induced by noxious gases and particles, such as oxidants and xenobiotics, play a major role in the onset and progression of the disease. An impairment of protective mechanisms of the lungs against such harmful molecules would also contribute to the disease process.

Oxidative stress and imbalances in the host defense mechanisms appear to be among the causes of COPD. Reactive oxygen/nitrogen species have been suggested to be involved in the pathophysiology of COPD through several studies analyzing airway samples such as induced sputum [8,9]. It also has been reported that antioxidant enzyme activities are altered in COPD. In the present study, the mRNA expression for catalase, a catalyzing enzyme for the clearance of hydrogen peroxide, was decreased in the peripheral lungs from COPD subjects. It has been reported that catalase activity is decreased in circulating red blood cells in patients with COPD [10], while the level of catalase is often increased in those from smokers, probably due to upregulation as an adaptive response against oxidative stress by cigarette smoking [11]. Recently, Ning and colleagues have reported the decreased gene expression for catalase in surgically obtained lung tissue from patients with COPD, using a serial analysis of gene expression [12]. Our results are consistent with their result and further extend the findings by showing that the decrease in catalase mRNA expression in the peripheral lung tissue was correlated to the degree of airflow limitation in COPD patients. These results suggest that impaired protective activity of catalase against oxidative stress may lead to enhanced pathophysiological changes in COPD.

Other protective mechanisms against toxic substrates involve xenobiotic-metabolyzing enzymes such as GSTP1, GSTM1 and mEPHX, the mRNA expressions of which were decreased in COPD patients in the present study. GSTP1 and GSTM1 are members of the glutathione *S*-transferase family known as antioxidative xenobiotic enzymes. GSTP1 and GSTM1 are expressed in alveoli, alveolar macrophages and respiratory bronchioli in the peripheral lung [13], and play an important role in the detoxification of xenobiotics which are contained in cigarette smoke and occupational and environmental pollutants. GSTP1 has been reported to exert a protective effect against cigarette smoke extract in human lung fibroblasts in vitro [14]. mEPHX is strongly expressed in bronchial epithelial cells in the lung [15], and is involved in the first-pass metabolism of highly reactive epoxide intermediates. Several genetic researches also have suggested that polymorphisms of GSTP1, GSTM1 and mEPHX genotypes may associate with a susceptibility to COPD or the severity of the disease in some races including Japanese [16–18].

Also, members of the TIMP family exert inhibitory activities against matrix metalloproteinases the involvement of which in the disease process of COPD has been suggested [19]. Recently, it has been reported that polymorphisms of the TIMP2 gene are associated with COPD susceptibility in a Japanese population suggesting a relationship between the decreased activity of TIMP2 and the pathogenesis of the disease [20]. It has been also reported that TIMP2 mRNA expression is decreased in the lung tissue from severer COPD subjects [12]. Our data are in line with these reports.

In the present study, decreased mRNA expressions for catalase, GSTP1 and mEPHX were evident in the peripheral lung tissue in COPD. The degree of the decrease in the mRNA expressions for these antioxidant and xenobiotic enzymes significantly correlated with the degree of airflow limitation, %FVC, %FEV_1, FEV_1/FVC%, V_{50}/HT

Table 3
mRNA expressions of the 42 studied candidate genes

	Non-COPD		COPD
	Never smoked	Smokers	
Genes that are significantly downregulated in COPD versus non-COPD			
Catalase	0.251±0.025	0.233±0.026	0.165±0.013**,†
GSTP1	0.647±0.069	0.554±0.028	0.449±0.027*,†
mEPHX	0.228±0.028	0.223±0.014	0.153±0.014*,††
GSTM1	0.136±0.033	0.229±0.044*	0.126±0.019†
TIMP2	0.873±0.166	1.135±0.101*	0.870±0.110†
Genes that are significantly upregulated in COPD versus non-COPD			
IL-1β	0.051±0.021	0.105±0.037	0.203±0.058**
IL-8	0.071±0.036	0.237±0.093	0.618±0.188**
Gro-α	0.002±0.001	0.009±0.006	0.015±0.005**
MCP-1	0.286±0.120	0.739±0.255	1.299±0.316**
Genes where no significant change is observed in COPD versus non-COPD			
XDH	0.001±0.000	0.001±0.000	0.001±0.000
HMOX1	0.133±0.020	0.211±0.040	0.333±0.083
GPX3	0.736±0.178	1.030±0.117	0.831±0.206
GSTT1	0.013±0.006	0.022±0.012	0.023±0.008
SOD1	0.058±0.008	0.047±0.010	0.045±0.007
SOD2	0.384±0.090	0.517±0.122	0.660±0.139
SOD3	0.221±0.018	0.234±0.013	0.187±0.013
MMP1	0.001±0.001	0.002±0.002	0.004±0.002
MMP2	0.649±0.113	0.867±0.161	0.816±0.091
MMP9	0.009±0.002	0.016±0.010	0.012±0.004
MMP12	0.002±0.001	0.002±0.001	0.002±0.001
ELA2	0.001±0.000	0.001±0.000	0.001±0.000
TIMP1	0.545±0.148	0.766±0.240	0.931±0.178
SERPINA1	0.529±0.062	0.685±0.084	0.804±0.085
SLPI	0.524±0.173	0.354±0.089	0.293±0.050
IL-13	0.001±0.001	0.001±0.001	0.003±0.001
TNF-α	0.019±0.003	0.034±0.005	0.034±0.006
TNF-α R	0.114±0.013	0.146±0.011	0.131±0.011
IFN-γ	0.001±0.001	0.002±0.001	0.001±0.000
IL-17	0.000±0.000	0.000±0.000	0.000±0.000
GM-CSF	0.000±0.000	0.000±0.000	0.000±0.000
IL-10	0.004±0.001	0.007±0.002	0.006±0.001
BLT1	0.004±0.000	0.004±0.000	0.003±0.000
BLT2	0.001±0.000	0.002±0.000	0.001±0.000
VEGF	0.412±0.053	0.491±0.039	0.397±0.047
MUC5AC	0.011±0.010	0.004±0.004	0.000±0.000
MUC5B	0.001±0.001	0.001±0.001	0.001±0.000
MUC8	0.001±0.000	0.001±0.000	0.001±0.000
CTSL	0.118±0.019	0.099±0.014	0.134±0.018
TGF-β	0.121±0.013	0.161±0.012*	0.159±0.012
EGF	0.000±0.000	0.000±0.000	0.001±0.000
IL-4	0.000±0.000	0.000±0.000	0.000±0.000
IL-5	0.000±0.000	0.000±0.000	0.001±0.000

The data are expressed as mean±SEM.
*$P<0.05$, **$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney U-test).
†$P<0.05$, ††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney U-test).

and V_{25}/HT. These data suggest that impairments in the activities of these enzymes protective against oxidants and xenobiotics could contribute to the pathophysiological changes in COPD. Since reactive xenobiotics also inhibit antiproteinases and increase proteinase secretion from neutrophils, a combination of oxidative stress and an imbalance of proteinase/antiproteinases may aggravate the airway inflammation and tissue disruption in COPD. On the other hand, the expressions of mRNA for GSTM1 and TIMP2 were significantly increased in lung tissues from non-COPD smokers compared with those from non-COPD subjects who never smoked, but were not increased in lung tissues from COPD subjects. These changes may imply that GSTM1 and TIMP2 could be upregulated due to cigarette smoking as adaptive responses and that these protective mechanisms could be impaired in COPD lungs leading to the development of the disease.

Decreased mRNA expression levels of catalase, GSTP1 and mEPHX, but not GSTM1 and TIMP2, were associated with cigarette smoking history assessed as the amount of cigarettes smoked (pack-years). The relationships between cigarette smoke exposure and the expressions of these enzymes are uncertain. However, some studies have suggested that cigarette smoke exposure may decrease the activities or expressions of catalase and GSTP1. The catalase activity in erythrocytes was decreased in cigarette smokers compared with non-smokers, and was slowly increased in smokers after smoking cessation [21]. The activity of GST in lung tissue was reduced by cigarette smoking [22]. In another study, ongoing cigarette smoke exposure correlated with depressed levels of GSTP1 mRNA expression in buccal cells [23]. Also, cigarette smoke exposure to a previously tobacco-naïve subject induced a transient decline of GSTP1 mRNA expression in such cells [23]. As for mEPHX, however, its activity and mRNA expression have been reported so far to be not affected in rat tissues including lungs [24] or enhanced in the lungs of patients with lung cancer [22] by cigarette smoke exposure. In those studies, the changes of mEPHX activity might have resulted from the relatively short-term effect of cigarette smoke exposure. The negative correlation between the mEPHX mRNA expression in the peripheral lung tissues and cigarette smoking in the present study may suggest a long-term effect of cigarette smoke exposure on the mEPHX expression in the lungs. The mechanisms of the decreases in the mRNA expressions for catalase, GSTP1 and mEPHX in the peripheral lung tissue by the effects of cigarette smoke are unknown. Further investigation that addresses this issue may increase our understanding about the cigarette smoke-induced processes by which the protective mechanisms against toxic substrates in the lungs become impaired.

In the present study, we found elevated expression levels of mRNA for several inflammatory cytokines and chemokines, IL-1β, IL-8, Gro-α and MCP-1, in the lungs from COPD subjects. The cellular inflammatory response in COPD is dominated by neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. It has been reported that increased activities of inflammatory cytokines and chemokines related to these inflammatory cells are associated with COPD and cigarette smoke exposure

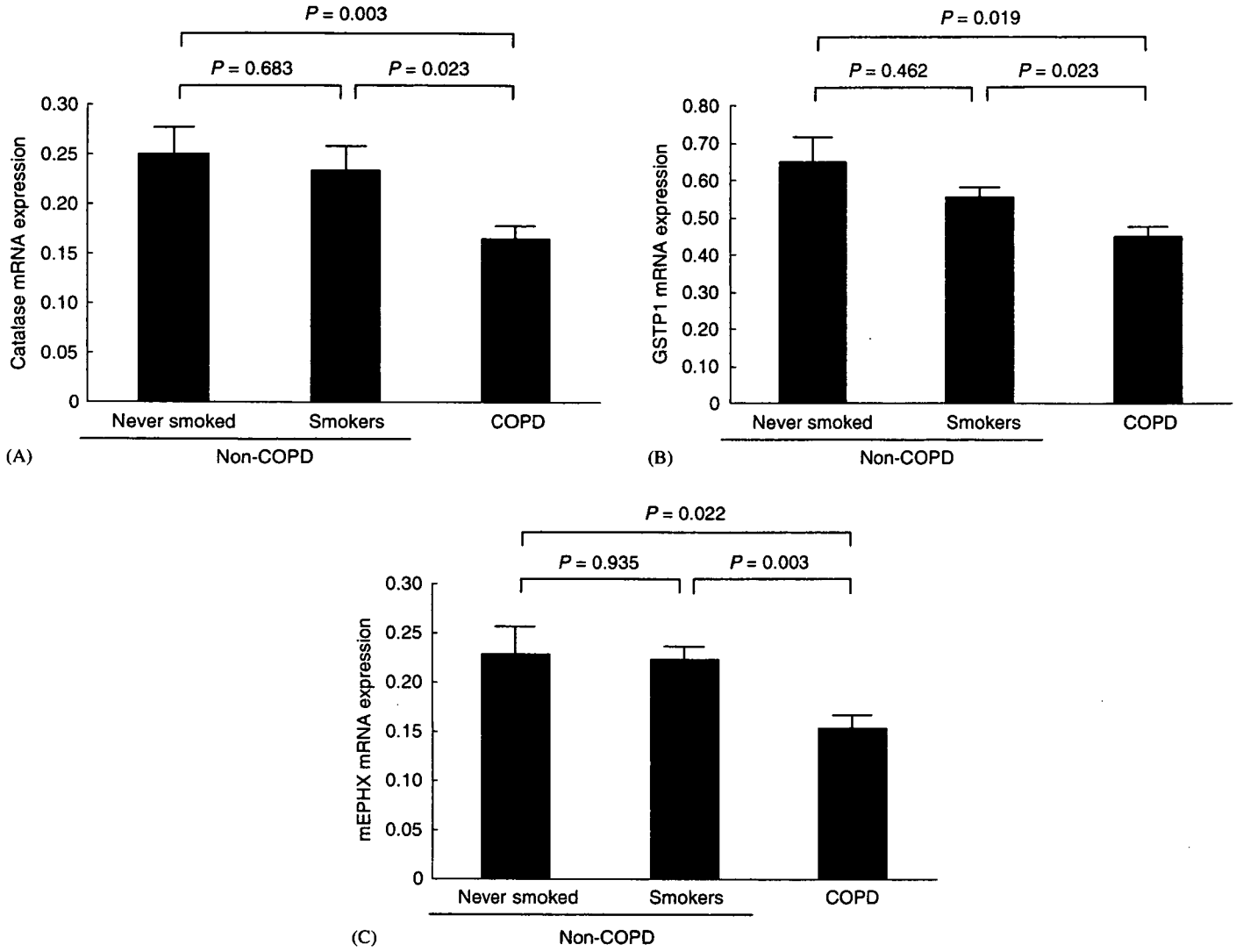

Fig. 1. mRNA expression for catalase (plate A), GSTP1 (plate B) and mEPHX (plate C) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean ± SEM. *P* values compared between two groups (Mann–Whitney *U*-test).

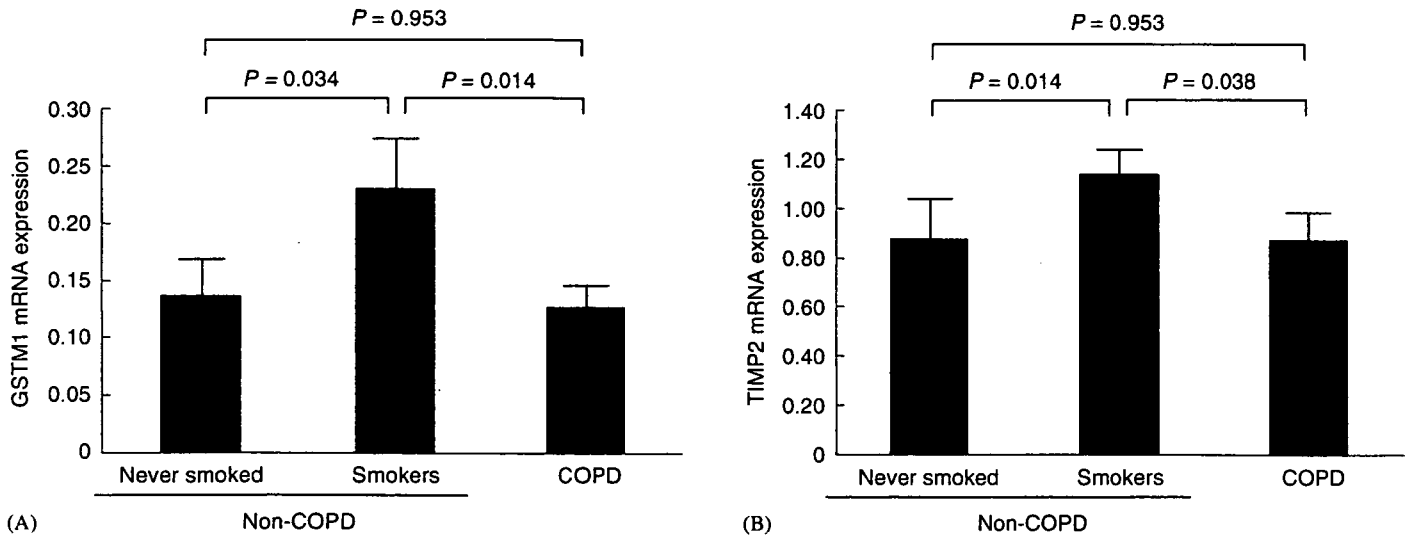

Fig. 2. mRNA expression for GSTM1 (plate A) and TIMP2 (plate B) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean ± SEM. *P* values compared between two groups (Mann–Whitney *U*-test).

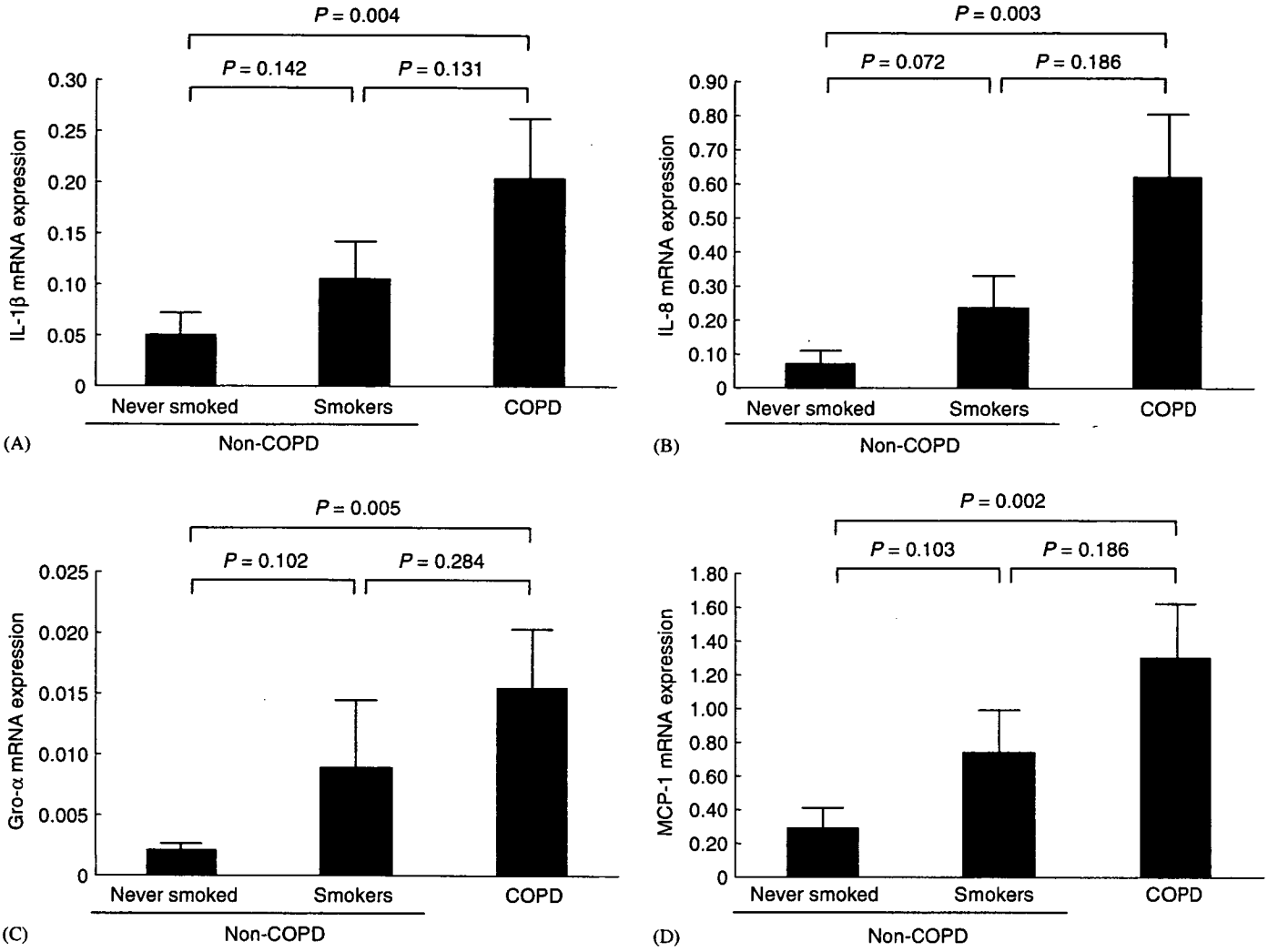

Fig. 3. mRNA expression for IL-1β (plate A), IL-8 (plate B), Gro-α (plate C) and MCP-1 (plate D) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean$\pm$SEM. P values compared between two groups (Mann–Whitney U-test).

[25–30]. Our results are consistent with these previous reports suggesting the involvement of these cytokines in the disease process of COPD.

Although the present study revealed significant changes in the mRNA expressions for several molecules in the whole tissue of the peripheral lungs of COPD subjects, the exact cellular sites of the altered mRNA expressions for individual genes are unknown. As for the chemokines, an analysis of lung tissue using an in situ hybridization technique has revealed higher mRNA and protein expression levels for IL-8 and MCP-1 in bronchiolar epithelium in COPD subjects compared to those in smokers without COPD [31]. Recently, Fuke et al. [32] have reported that the expressions of mRNA for IL-8 and MCP-1 are elevated in bronchiolar epithelial cells but not in alveolar macrophages by means of a laser-capture microdissection technique. These results suggest that the increased expression levels of IL-8 and MCP-1 in the peripheral lung tissue found in the present study may be due to the increased expression in epithelial cells.

The limitations of this study are lack of sex and age matching among the groups and lack of smoking history matching between the groups who had cigarette smoking histories. Cigarette smoking history assessed as pack-years positively or negatively correlated with the mRNA expressions for some enzymes and cytokines mRNA expressions. The data suggest that cigarette smoke exposure affects the disease process of COPD, but do not provide an answer for the question of why only some smokers develop COPD. Recently, two studies reported the altered expressions of a number of genes in epithelial cells from smokers [33] and in lung tissues from COPD subjects [12]. Including the present study, three studies have analyzed gene expressions in the lungs of COPD subjects or smokers, but the profiles of the subjects differed in terms of the disease severity and smoking status among the study groups. The collection of data acquired from a large population having differences in disease severity and smoking profiles is needed to further elucidate the mechanisms of disease onset and progression.

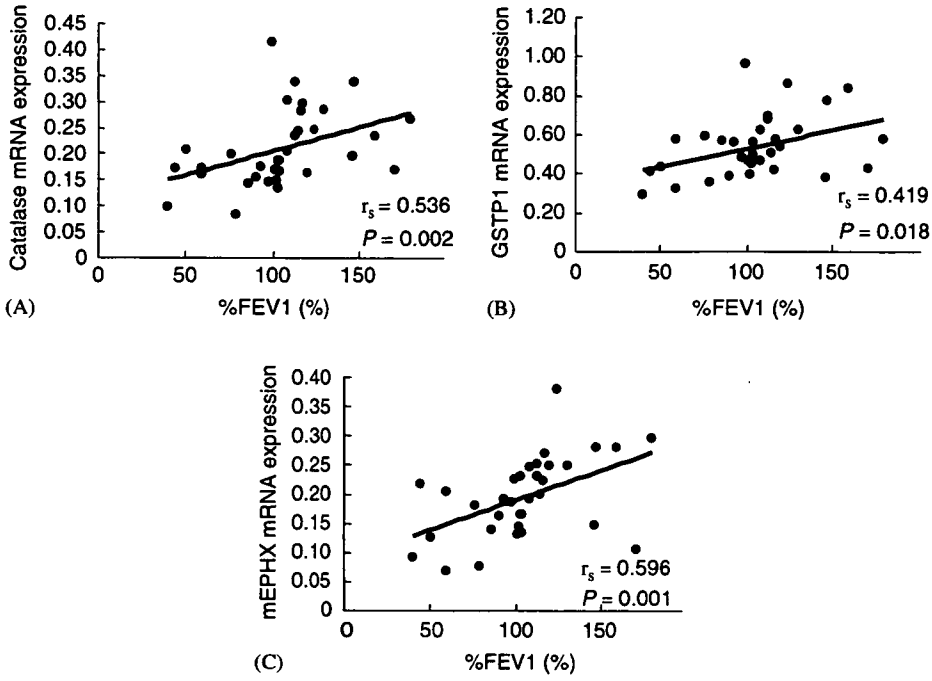

Fig. 4. Relationships between mRNA expressions for catalase (plate A), GSTP1 (plate B) and mEPHX (plate C) and the degree of airflow limitation. Relationship was analysed by Spearman's rank correlation test. A correlation coefficient of Spearman's rank correlation test (r_s) and P-value are indicated.

Table 4
Correlation coefficients of Spearman's rank correlation test (r_s) for relationships between mRNA expression and lung function, smoking history

	Catalase	GSTP1	mEPHX	IL-1β	IL-8	Gro-α	MCP-1
%FVC	0.457**	0.368*	0.495**	−0.297	−0.320	−0.349*	−0.262
%FEV_1	0.536**	0.419*	0.596**	−0.316	−0.329	−0.342	−0.280
FEV_1/FVC%	0.433*	0.411*	0.423*	−0.317	−0.398*	−0.392*	−0.330
V_{50}/HT	0.420*	0.368*	0.402*	−0.357*	−0.383*	−0.375*	−0.373*
V_{25}/HT	0.432*	0.354*	0.442*	−0.264	−0.328	−0.358*	−0.277
Smoking (pack-year)	−0.518**	−0.514**	−0.399*	0.567**	0.511**	0.573**	0.561**

r_s is a correlation coefficient of Spearman's rank correlation test.
*$P<0.05$.
**$P<0.01$ (Spearman's rank correlation test).

In summary, we found decreased mRNA expressions for catalase, GSTP1, GSTM1, mEPHX and TIMP2 and increased mRNA expressions for IL-1β, IL-8, Gro-α and MCP-1 in peripheral lung tissues from COPD subjects. Most of these changes were associated with the degree of airflow limitation and with cigarette smoking habit. The impairment of protective mechanisms against oxidants and xenobiotics as well as upregulation of CXC- and CC-chemokines seems to be associated with cigarette smoking and to be involved in the pathogenesis of COPD. Interventions targeting these molecules may provide a possible strategy for modifying the disease process of COPD.

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References

[1] Murray CJ, Lopez AD. Evidence-based health policy–lessons from the global burden of disease study. Science 1996;274:740–3.