

Oxidative stress overproduction and intracellular signaling pathway activation are the major pathways in the core-induced liver pathology

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.^{10,17} On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This finding reflects a state of overproduction of reactive oxygen species (ROS) in the liver,¹⁸ or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.^{19,20} The overproduction of oxidative stress results in the generation of deletions in mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice: hemoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione S-transferase levels are increased and enhanced by iron overloading (Moriya et al., manuscript in preparation). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the antioxidant systems, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that can no longer be scavenged by a physiological antagonistic system. This idea suggests that the inflammation in chronic HCV infection would have a characteristic difference in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.^{10,19} The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.²¹

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)- α and interleukin-1 β have been found to be transcriptionally activated.²² The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model before HCC development, only the JNK route is activated. Downstream of JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.^{20,21} At far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage for cell proliferation to the hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28 γ (PA28 γ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance, and HCC.^{23,24}

Lipid metabolism and HCV infection

Steatosis is frequently observed in chronic hepatitis C patients and is significantly associated with increased fibrosis and progression rate of fibrosis of the liver.⁶ A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly upregulated or downregulated (Table 1).

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver resulting from simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased; this is also the case in the comparison of liver tissues from hepatitis C patients and patients with simple fatty liver due to obesity.²⁰ The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are at least three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1.²⁵ Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of

Table 1. Cellular genes differentially expressed in hepatitis C virus (HCV) core transgenic mouse liver

	Upregulated	Downregulated
Lipid metabolism	NPC1 Catalase Very long chain acyl-CoA dehydrogenase Carboxylesterase selenoprotein P Carbonic anhydrase Adipose differentiation-related protein Bilirubin/phenol family UDP glucuronosyltransferase	Stearoyl-CoA desaturase Sterol-carrier protein X Alpha-enolase carnitine acetyltransferase Gal beta 1,4(3) GlcNAc alpha 2,3-sialyltransferase Very long chain acyl-CoA synthetase Liver transferrin 4-Hydroxyphenylpyruvate dioxygenase LAF1 transketolase s-Adenosylmethionine synthetase Apolipoprotein A-II Human guanine nucleotide regulatory protein Alpha-fetoprotein Retinol binding protein
Transcription and cell proliferation	Int-6 GCN5L1 <i>H. sapiens</i> 8.2k-Da differentiation factor USF1 Initiation factor eIF-4A1 Human elongation factor-1-delta Sui1	
Inflammation	Alpha-1 protease inhibitor 3 Hemopexin	Alpha-2-macroglobulin LMW prekinogen Complement component C3 AHSG(alpha 2 HS-glycoprotein) homologue Vitronectin Epithelin 1 and 2 Murinoglobulin
Others	Microvascular endothelial differentiation gene 1 Diazepam-binding inhibitor Argininosuccinate synthetase Skeletal muscle alpha-tropomyosin Ampd3 gene DNA-binding protein	

microsomal triglyceride transfer protein (MTP) by HCV core protein²⁶; this inhibits the secretion of very low density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last pathway involves sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, whereas neither SREBP-2 nor SREBP-1a is upregulated.²⁷

In relation to lipid metabolism, the core protein has also been found to interact with retinoid X receptor (RXR)- α .²⁸ RXR- α is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors, including PPAR (peroxisome proliferator-activated receptor)- α , and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation, and proliferation. In fact, the core protein of HCV activates genes that have an RXR- α -responsive element as well as those with a PPAR- α -responsive element, both in mice and in cultured cells.²⁸ Based on these results, we, then, examined the expression and function of PPAR- α in the liver of core gene transgenic mice.

PPAR- α activation in HCV-associated hepatocarcinogenesis

PPAR- α , one of the PPAR genes, plays a central role as a heterodimer with RXR- α in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.²⁹ On the other hand, prolonged administration of PPAR- α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.²⁹

The level of PPAR- α protein was increased in the liver of core gene transgenic mice as early as 9 months of age. PPAR- α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR- α mRNA was not increased at any age. By pulse-chase experiment, the stability of nuclear PPAR- α was increased in the presence of the core protein. In line with the increase of PPAR- α protein, target genes of PPAR- α were activated in the liver of core gene transgenic mice; these genes include

cyclin D1, cyclin-dependent kinase (CDK)-4, acy-CoA oxidase, and peroxisome thiolase.³⁰ However, in general, the activation of PPAR- α leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR- α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR- α activation in pathogenesis of steatosis and HCC, we mated a core gene transgenic mouse with a PPAR- α knockout (KO) mouse and studied the phenotype. PPAR- α KO mice have reduced expression of target genes of PPAR- α , and have mild steatosis in the liver, as expected.³¹ It was unanticipated, however, that steatosis was absent in PPAR- α -null or -heterozygous core gene transgenic mice but present in PPAR- α -intact core gene transgenic mice at the age of 9 or 24 months.³⁰ 8-Hydroxy deoxyguanosine (8-OHdG) and peroxy lipids, both of which are markers for oxidative stress, were decreased in PPAR- α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to overproduction of oxidative stress,¹⁹ was also improved in PPAR- α KO core gene transgenic mice.

Finally, PPAR- α KO core gene transgenic mice did not develop HCC at the age of 24 months, whereas about one-third of PPAR- α -intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR- α gene also did not develop HCC.³² When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR- α -heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core gene (+) mice with greater PPAR- α activation. It should be noted that steatosis was present only in core gene (+) PPAR- α -heterozygous mice. In summary, steatosis and HCC developed in PPAR- α -intact but not in PPAR- α -heterozygous or PPAR- α -null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR- α would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR- α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR- α may exacerbate steatosis. Persistent activation of PPAR- α with "strong" ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

HCV core protein causes "fatty acid spiral"

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV

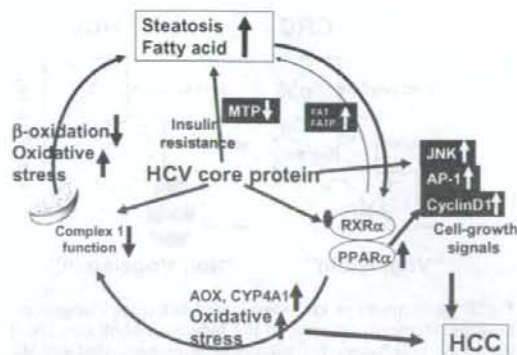


Fig. 2. "Fatty acid spiral" by HCV core protein. In HCV infection, the core protein induces steatosis via several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR- α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. *HCV*, hepatitis C virus; *HCC*, hepatocellular carcinoma; *ROS*, reactive oxygen species; *JNK*, c-Jun N-terminal kinase; *ERK*, extracellular signal-regulated kinase; *AP-1*, activating protein-1; *RXR- α* , retinoid X receptor- α ; *PPAR- α* , peroxisome proliferator activated receptor- α ; *AOX*, acyl-CoA oxidase; *CYP*, cytochrome P450; *MTP*, microsomal triglyceride transfer protein; *FAT*, fatty acid translocase; fatty acid transport protein.

infection. However, in HCV infection, the core protein induces steatosis through the aforementioned pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR- α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical-type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

The HCV protein may allow some steps in multistep hepatocarcinogenesis to be skipped

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory outlined by Kinzler and Vogelstein³³ has gained wide popularity. They have proposed that the

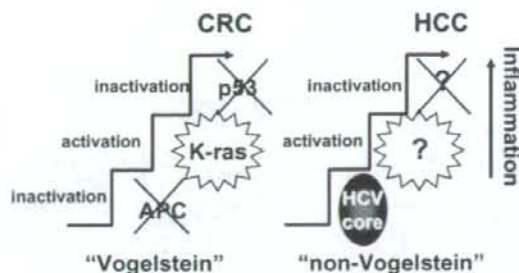


Fig. 3. Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers

development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in K-ras for activation, and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.³³ Their theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 3).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (see Fig. 3). The overall effect achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.³⁴ Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the nonmetastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

References

- Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990;87:6547-9.
- Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998;28:930-8.
- Okuse C, Yotsuyanagi H, Koike K. Hepatitis C as a systemic disease: virus and host immunologic responses underlie hepatic and extrahepatic manifestations. *J Gastroenterol* 2007;42:857-65.
- Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J Gastroenterol* 2005;40:329-36.
- Negro F. Insulin resistance and HCV: will new knowledge modify clinical management? *J Hepatol* 2006;45:514-9.
- Powell EE, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. *Hepatology* 2005;42:5-13.
- Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671-5.
- Yotsuyanagi H, Shintani Y, Moriya K, Fujie H, Tsutsumi T, Kato T, et al. Virological analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. *J Infect Dis* 2000;181:1920-8.
- Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997;78:1527-31.
- Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Matsuura Y, et al. Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065-8.
- Koike K, Moriya K, Ishibashi K, Matsuura Y, Suzuki T, Saito I, et al. Expression of hepatitis C virus envelope proteins in transgenic mice. *J Gen Virol* 1995;76:3031-8.
- Koike K, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, et al. Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997;94:233-6.
- Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992;15:572-7.
- Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002;122:352-65.
- Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, et al. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. *J Gen Virol* 2005;86:2185-96.
- Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006;80:7199-207.
- Moriya K, Fujie H, Yotsuyanagi H, Shintani Y, Tsutsumi T, Matsuura Y, et al. Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. *Jpn J Med Sci Biol* 1997;50:169-77.
- Sasaki Y. Does oxidative stress participate in the development of hepatocellular carcinoma? *J Gastroenterol* 2006;41:1135-48.
- Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. *Cancer Res* 2001;61:4365-70.

20. Moriya K, Todoroki T, Tsutsumi T, Fujie H, Shintani Y, Miyoshi H, et al. Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biophys Biochem Res Commun* 2001;281:1207-12.
21. Okuda M, Li K, Beard MR, Showalter LA, Schole F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002;122:366-75.
22. Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, et al. Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. *Virology* 2002;304:415-24.
23. Tsutsumi T, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, et al. Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003;38:820-8.
24. Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, et al. Hepatitis C virus core protein induces insulin resistance through a PA28 γ -dependent pathway. *J Virol* 2007;81:1727-35.
25. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Kimura S, Moriya K, et al. Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004;126:840-8.
26. Perlemuter G, Sabile A, Letteron P, Vona G, Topilko A, Koike K, et al. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002;16:185-94.
27. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28 γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci USA* 2007;104:1661-6.
28. Tsutsumi T, Suzuki T, Shimoike T, Moriya K, Yotsuyanagi H, Matsuura Y, et al. Interaction of hepatitis C virus core protein with retinoid X receptor- α modulates its transcriptional activity. *Hepatology* 2002;35:937-46.
29. Peters JM, Cheung C, Gonzalez FJ. Peroxisome proliferator-activated receptor- α and liver cancer: where do we stand? *J Mol Med* 2005;83:774-85.
30. Tanaka N, Moriya K, Kiyosawa K, Koike K, Aoyama T. Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: implications for HCV-associated hepatocarcinogenesis. *Int J Cancer* 2008;122:124-31.
31. Akiyama TE, Sakai S, Lambert G, Nicol CJ, Matsusue K, Pimprale S, et al. Conditional disruption of the peroxisome proliferator-activated receptor gamma gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux. *Mol Cell Biol* 2002;22:2607-19.
32. Tanaka N, Moriya K, Kiyosawa K, Koike K, Gonzalez FJ, Aoyama T. PPAR- α is essential for severe hepatic steatosis and hepatocellular carcinoma induced by HCV core protein. *J Clin Invest* 2008;118:683-94.
33. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87:159-70.
34. Koike K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. *Clin Gastroenterol Hepatol* 2005;3:S132-5.

A Single Amino Acid of Toll-like Receptor 4 That Is Pivotal for Its Signal Transduction and Subcellular Localization*

Received for publication, April 22, 2008, and in revised form, October 29, 2008. Published, JBC Papers in Press, December 8, 2008, DOI 10.1074/jbc.M803086200

Shintaro Yanagimoto^{2,5*}, Keita Tatsuno⁶, Shu Okugawa⁵, Takatoshi Kitazawa⁵, Kunihisa Tsukada⁵, Kazuhiko Koike⁵, Tatsuhiko Kodama⁶, Satoshi Kimura¹, Yoshikazu Shibasaki^{1,2}, and Yasuo Ota^{1,2}

From the ¹Center for Structuring Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8903, the ²Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, the ³Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Meguro-ku, Tokyo 153-8904, the ⁴Tokyo Teishin Hospital, Fujimi, Chiyoda-ku, Tokyo 102-8798, and the ⁵Department of Medicine, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-ku, Tokyo 173-8605, Japan

Toll-like receptor 4 (TLR4) is essential for recognizing a Gram-negative bacterial component, lipopolysaccharide (LPS). A single amino acid mutation at position 712 of murine TLR4 leads to hyporesponsiveness to LPS. In this study we determined that an amino acid, a leucine at position 815 of human TLR4, is also pivotal for LPS responsiveness and subcellular distribution. By replacing the leucine with alanine, the mutant TLR4 lost responsiveness to LPS and did not localize on the plasma membrane. In addition, it does not coprecipitate with myeloid differentiation-2, an accessory protein that is necessary for TLR4 to recognize LPS. These results suggest that the leucine at position 815 is required for the normal maturation of TLR4 and for formation of the TLR4-MD-2 complex.

Toll-like receptors (TLRs)³ play essential roles in both innate and adaptive immunity (1). Thirteen members of the TLR family have been identified in mammals. TLRs have leucine-rich-repeats in their extracellular domains and a Toll/Interleukin-1 receptor (TIR) in their cytoplasmic domains, the latter of which mainly mediates intracellular signaling. Signaling pathways of TLRs, except for TLR3, depend on an adapter protein, MyD88 (myeloid differentiation factor 88), which interacts with the TIR domain of TLRs. This pathway leads to the activation of the transcription fac-

tor NF- κ B and production of cytokines such as tumor necrosis factor- α and interleukin-6. Another important signaling pathway mediated by TLR3 and TLR4 that exploits the TIR domain is the MyD88-independent pathway. This pathway involves different adapter proteins, such as the TIR domain-containing adaptor inducing interferon- β (TRIF) and TRIF-related adaptor molecule (2–4), and is essential for production of type I interferon through activation of interferon regulatory factor-3.

TLRs recognize as ligands several microbial pathogen-associated molecular patterns. One such pathogen-associated molecular pattern is lipopolysaccharide (LPS), which is recognized by TLR4. LPS triggers severe immunologic reactions by the host in Gram-negative bacterial infections and has drawn attention in many clinical situations. TLR4 is the first mammalian TLR to be discovered in the context of immunology. TLR4 was identified in the search for the genes responsible for LPS hyporesponsiveness (5, 6). The defect was found to stem from a single amino acid mutation, replacement of proline with histidine at position 712, in the cytoplasmic tail of murine TLR4. The study led to the discovery of the importance of TLR4 in innate immunity.

A variety of cells are activated by LPS stimulation through TLR4. TLR4 forms a receptor complex with an accessory protein, myeloid differentiation-2 (MD-2). MD-2 first associates with TLR4 in the endoplasmic reticulum (ER) and *cis*-Golgi, and both proteins move together to the plasma membrane (7, 8). Upon recognition of LPS, the TLR4-MD-2 complex receives LPS on the cell surface and initiates intracellular signaling. The expression of TLR4 in the absence of MD-2 does not confer full responsiveness to LPS stimuli in experimental cell lines (9). An analysis of MD-2 knockout mice revealed that MD-2 is important not only for LPS sensing but also for cellular distribution of TLR4.

In this study we hypothesized that the cytoplasmic tail of TLR4 contains regions that control both localization and signaling. Using truncation and mutation analysis, and paying particular attention to the TIR domain, we identified a single amino acid that is pivotal for both TLR4 signaling and subcellular distribution. The site we found was on the C-terminal portion of the TIR domain for which no specific function has been yet determined.

* This work was partly supported by the Program of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, by the Focus 21 project of the New Energy and Industrial Technology Development Organization, and by the Special Coordination Fund for Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology. This study was also partly supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology (to Y. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed. Tel: 81-3-3964-1211 (ext. 1756); Fax: 81-3-3579-6310; E-mail: yasuo-ota@umin.ac.jp.

³ The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/Interleukin-1 receptor; TRIF, TIR domain-containing adaptor inducing interferon- β ; LPS, lipopolysaccharide; MD-2, myeloid differentiation-2; ER, endoplasmic reticulum; GFP, green fluorescent protein; EGFP, enhanced GFP; RLA, relative luciferase activity; Sulfo-NHS-SS-Biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopyronate.

An Important Amino Acid of TLR4 for Its Function

EXPERIMENTAL PROCEDURES

Reagents and Other Materials—Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was purchased from Sigma-Aldrich and applied without purification. FLAG- and hexa-histidine (His₆)-tagged human TLR4 expression plasmid (pEFBOS/humanTLR4flaghis) and FLAG- and His₆-tagged human MD-2 expression plasmid (pEFBOS/humanMD-2flaghis) were generous gifts from Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo, Japan). Human CD14 cDNA plasmid (pCMV6-XL5/humanCD14) was purchased from OriGene (Rockville, MD). Fluorescent protein expression vector pEGFP-N3 was purchased from Clontech (Mountain View, CA). Anti-TLR4 monoclonal antibody (clone HTA125) was purchased from Abcam (Cambridge, MA). Anti-FLAG monoclonal antibody (clone M2) was purchased from Sigma-Aldrich. Anti-A.v. (GFP) monoclonal and polyclonal antibodies were purchased from Clontech. Control immunoglobulins for immunoprecipitation were purchased from BD Biosciences (San Jose, CA). Horseradish peroxidase-labeled anti-immunoglobulins antibodies were purchased from Dako (Glostrup, Denmark). BlockAce (DS Pharma Biomedical, Osaka, Japan) solution was used as blocking buffer for Western blotting.

Cell Culture—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum supplemented with penicillin-streptomycin solution (Invitrogen). FuGENE 6 transfection reagent (Roche Applied Science) was used for transient cotransfection according to the manufacturer's instructions. Culture dishes or plates were prepared to 70% confluence prior to transfection. Cells were used for experiments 36 h later. The transfection conditions were optimized for microscopic observation of the expressed fluorescent protein and were kept unchanged in other experiments.

Expression Vector Subcloning and Mutagenesis—Wild-type TLR4 cDNA was excised from pEFBOS/humanTLR4flaghis and subcloned into pEGFP-N3 so that when expressed enhanced green fluorescent protein (EGFP) would be fused at the C terminus of TLR4 (pEGFP-N3/humanTLR4). All mutations were introduced into pEFBOS/humanTLR4flaghis and pEGFP-N3/humanTLR4 using the QuikChange site-Directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and were confirmed by sequencing. For the truncation analysis, two identical unique restriction sites were prepared in the TLR4-coding region of pEFBOS/humanTLR4 using a QuikChange kit, and the DNA fragment to be removed, which was a part of the C terminus of TLR4, was excised enzymatically. After agarose gel purification, the linear double-stranded DNA was ligated to re-form a circular plasmid. Restriction sites were designed so as not to cause a frameshift between TLR4 and EGFP.

Confocal Laser Scanning Microscopy of Cells—Samples were fixed in 3% paraformaldehyde-phosphate-buffered saline at 37 °C for 10 min. Fluorescence images of fixed samples were recorded using a Fluoview FV1000 Confocal Microscope (an inverted confocal laser scanning microscope, Olympus, Tokyo, Japan).

Immunoprecipitation—Transfected cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 1 mM 1,4-dithiothreitol, and proteinase inhibitor mixture), sonicated, and centrifuged at 4 °C. Antibody was added to the supernatant, and the sample was rotated 1 h at 4 °C followed by the addition of protein G-Sepharose (GE Healthcare Life Sciences, Piscataway, NJ) and an additional 8-h incubation at 4 °C. Bound protein was washed three times in lysis buffer. Proteins were eluted by boiling in SDS sample buffer.

Biotinylation and Purification of Cell Surface Proteins—Prior to surface biotinylation, HEK 293T cells plated in a 100-mm dish were transiently transfected as described above. Surface biotinylation and subsequent purification of biotinylated proteins were performed using a Cell Surface Protein Biotinylation and Purification Kit (Pierce) following the manufacturer's instructions. Briefly, membrane-impermeable sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) was added to cell monolayers in the culture dishes and covalently bound to amines in proteins exposed on the cell surface. The affinity resin that binds to the biotin end of Sulfo-NHS-SS-Biotin was used to collect the biotinylated proteins. Reduction by 1,4-dithiothreitol causes cleavage of the disulfide bond in Sulfo-NHS-SS-Biotin, and the elute contains the biotinylated cell surface proteins. Each final sample obtained was considered to contain proteins from an equal amount of cells, because all culture plates were treated equally and grown to full confluence. All samples were sonicated and subjected to SDS-PAGE and Western blotting. The membrane to which protein was transferred was blocked in blocking buffer for 1 h. Then the membrane was incubated with a primary antibody, followed by incubation with horseradish peroxidase-labeled anti-immunoglobulins antibody. The protein bands were then visualized by using a chemiluminescence reagent, Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), according to the manufacturer's instructions.

Cell Stimulation Assays—HEK293T cells were plated and transiently transfected for assays. Thirty-six hours after the transfection, LPS was added to fresh culture medium in each well of the culture plates at the stated concentration. The duration of LPS stimulation was 7 h.

Dual Luciferase Reporter Assays for NF- κ B Activation—HEK293T cells were plated in 12-well culture plates (4×10^4 cells/well), and experimental cDNA plasmids were transiently transfected 36 h later using the FuGENE 6 transfection reagent with 0.5 μ g of NF- κ B reporter plasmid expressing firefly luciferase (pNF- κ B-Luc, Stratagene) and 0.05 μ g of constitutively active *Renilla* luciferase reporter plasmid (pRL-TK, Promega, Madison, WI) in addition to 0.5 μ g each of TLR4-EGFP plasmid and MD-2 plasmid. Stimulation experiments were performed 36 h later. Firefly luciferase and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and the Genelight55 luminometer (Microtech, Chiba, Japan). Relative luciferase activity (RLA) was obtained as the ratio of firefly luciferase activity to *Renilla* luciferase activity. Results are expressed as the ratio of RLA with LPS stimulation to RLA without LPS stimulation ($[\text{RLA LPS+}]/[\text{RLA LPS-}]$). This ratio should ideally approach 1 when no response to LPS stimulation is observed.

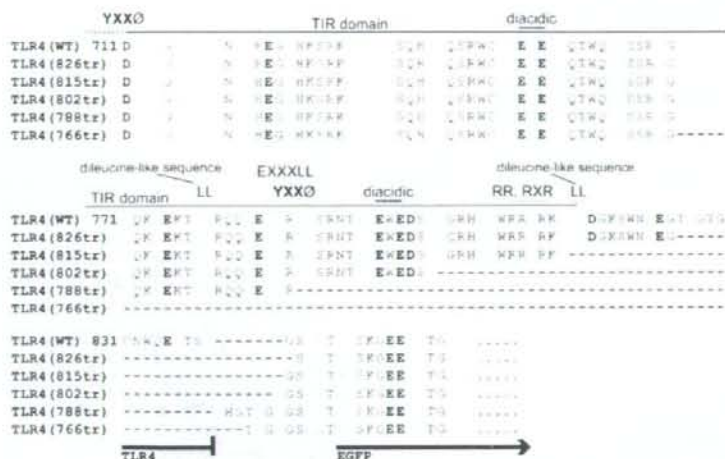


FIGURE 1. Alignment of the cytoplasmic domains of EGFP fusion TLR4 truncation mutants used in this study. TLR4 (766tr) signifies the mutant truncated at position 766. Others are named in the same manner. The amino acids are colored based on their physicochemical properties: pink, basic; blue, acidic; green, polar and neutral; and orange, hydrophobic. The black overline represents the TIR domain. Colored overlines indicate amino acid sequences identical to known sorting signal motifs except for two LLs, which are dileucine motif-like sequences in that they consist of solely two consecutive leucines without preceding aspartate or glutamate. Capital letters on the line signify the single-letter code for amino acids: E, glutamic acid; L, leucine; R, arginine; and Y, tyrosine. X signifies any amino acid, and Ø signifies an amino acid residue with a bulky hydrophobic side chain.

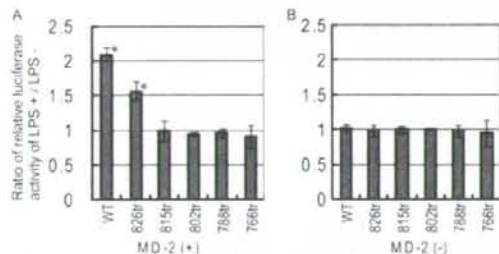


FIGURE 2. LPS responsiveness measured by NF- κ B luciferase assay. HEK293T cells were transfected with plasmids containing the gene for wild-type TLR4 or a truncated human TLR4-EGFP fusion protein, in addition to a luciferase reporter and human MD-2 plasmid (A) or unmodified plasmids (control) (B). After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. All results were expressed as the ratio of relative luciferase activity with LPS stimulation to that without stimulation. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$; TLR4 (826tr)-EGFP/MD-2 (+), $p = 0.016$).

Statistical Analyses—All quantitative experiments were repeated three times, and each experiment was done in triplicate. The ratio of relative luciferase activity of LPS+ to LPS- was calculated as the index of the responsiveness to the stimuli as explained above. When positive response is observed, the ratio should significantly exceed one. The means of the ratio were represented in *bar graphs*. The 95% confidence interval of the mean of the ratio was calculated and indicated on each *bar* in the *graph*, and p values were calculated using Student's t distribution compared with the hypothetical mean, one.

RESULTS

Truncation Analysis of TLR 4—To identify amino acid sequences in the cytoplasmic tail of TLR4 that are involved in

both signal transduction and subcellular distribution, first we generated five truncation mutants of TLR4 with a fluorescent protein (EGFP) at the C terminus of TLR4.

Although there are no known definite sorting signal motifs in the cytoplasmic tail of TLR4, some amino acid sequences are similar or identical to known general sorting signal motifs as shown in Fig. 1. YXXO, a form of tyrosine-based sorting signal, and EXXXLL, a form of dileucine (LL)-based sorting signal, both control protein internalization, lysosomal targeting, and basolateral targeting (10), where "X" stands for any amino acid, "Ø" stands for an amino acid residue with a bulky hydrophobic side chain, and other letters are single-letter abbreviations for the amino acids. "Diacidic" signals such as DXE mediate export from the ER (11). RR or RXR is another example of an ER export signal (12). Truncation sites were chosen so that some of these amino acid sequences were deleted in each mutant. Because the TIR domain, which is essential in TLR4 signaling and possibly subcellular localization (13), spans most of the cytoplasmic domain of TLR4, four out of five mutants have involvement in the TIR domain, which we hypothesized could result in impaired signal transduction and a change in subcellular distribution. Part of the cytoplasmic portion of the amino acid sequence of the truncation mutants is shown in Fig. 1. The five truncation mutant proteins lost their C-terminal tails at positions 826, 815, 802, 788, and 766, respectively, and were conjugated with EGFP *in vitro*. Actual truncation and ligation sites of all actual mutants were confirmed to have the designed DNA alignment by sequencing.

We utilized the luciferase reporter assay to assess NF- κ B transcription activity as an indicator of TLR4 response to LPS stimuli. MD-2 is reported to be essential for this response (9). However, because it is not known whether MD-2 is necessary for transduction of the truncated TLR4 signal as well, we performed the assays with and without MD-2. The index of cell responsiveness to the stimulation was measured as the ratio between RLA with LPS stimulation and RLA without LPS stimulation. Only cells transfected with TLR4 (826tr)-EGFP in combination with MD-2 retained responsiveness to LPS stimulation. One exception was wild-type TLR4-EGFP (Fig. 2A). HEK293T cells transfected with TLR4 but without MD-2 did not respond to LPS stimuli regardless of the TLR4-EGFP genotype (Fig. 2B).

Next, we compared the localization of wild-type and truncated mutants of TLR4-EGFP in HEK293T by fluorescence microscopy (Fig. 3A). The wild-type TLR4 cotransfected with MD-2 was expressed on the plasma membrane and also in the

An Important Amino Acid of TLR4 for Its Function

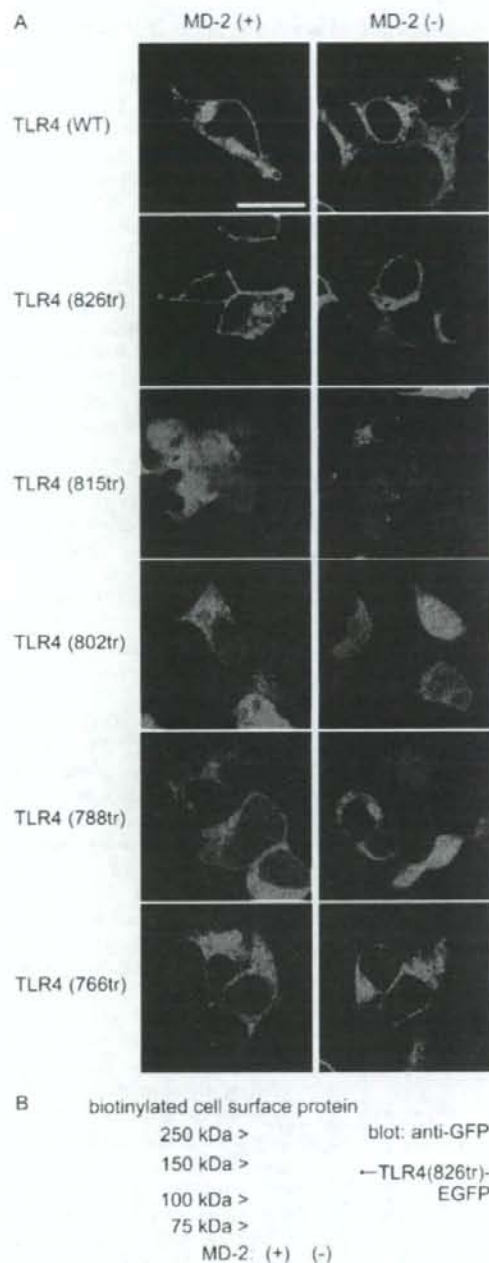


FIGURE 3. Residues 815–826 of TLR4 contain a region necessary for plasma membrane localization. *A*, cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Fluorescence from EGFP was observed in green. Each genotype of TLR4-EGFP was cotransfected with a human MD-2 plasmid or empty vector. *Bar*, 20 μ m. *B*, TLR4 (826tr)-EGFP with or without coexpression of MD-2 were tagged by biotinylation of the cell surface proteins and affinity-purified. TLR4 was visualized by immunoblotting using an anti-GFP monoclonal antibody. Samples from both combinations of DNAs were prepared from the same number of cells.

perinuclear area. These findings were consistent with observations by others (14, 15). TLR4 is reported to localize in the Golgi apparatus as well as on the plasma membrane. Our observation of TLR4-EGFP accumulation in the perinuclear area does not contradict the report that TLR4 partly localizes in the Golgi apparatus (14).

TLR4-EGFP truncation mutants, 815tr, 802tr, 788tr, and 766tr apparently did not localize at the plasma membrane. No particular fluorescence pattern that might be characteristic of localization to a specific intracellular compartment was observed. Only TLR4 (826tr)-EGFP, which has the shortest truncation, was expressed on the plasma membrane and in the perinuclear area, and the fluorescence pattern was similar to that of wild-type (Fig. 3*A*). No TLR4 genotypes, including wild-type TLR4-EGFP, clearly localized on the plasma membrane in the absence of MD-2 (Fig. 3*A*). MD-2 is reported to be necessary for localization of wild-type TLR4 at the plasma membrane (15), which is consistent with our observation. Intracellular distribution of mutant TLR4 varied depending on the genotype, but no particular cellular structure was identified as an alternative target site. Furthermore, we examined the plasma membrane expression of TLR4 (826tr)-EGFP by cell surface protein biotinylation. The expression level of TLR4 (826tr)-EGFP was markedly decreased without coexpression of MD-2 (Fig. 3*B*), which is compatible with the microscope observation.

Removal of the C-terminal segment of TLR4 at residue 826 does not qualitatively affect LPS responsiveness and subcellular distribution. However, when more residues, up to position 815, were removed, both signal transduction and plasma membrane localization were impaired. These results suggest that residues 815–826 of TLR4 contain at least one segment that is critical for those functions.

Amino Acid Sequence Replacement Analysis—To identify critical amino acid sequences in this region, we generated an amino acid replacement mutant of TLR4 instead of truncation mutants. As shown in Fig. 1, although it is not a canonical sequence, leucine-leucine at 815–816 partially fits a known sorting signal motif, a dileucine motif, (D/E)XXX(L/L) or DXXLL, which plays an important role in internalization of plasma membrane protein or sorting from the *trans*-Golgi network (10). Thus, as has been done in a similar study (16), a mutant was generated in which alanines were substituted for both leucines at positions 815 and 816.

We measured the NF- κ B activity of TLR4 (L815A/L816A)-EGFP, the mutant in which both leucines were replaced with alanines, under LPS stimulation (Fig. 4*A*). This mutant protein did not respond to LPS stimuli. Microscopic observation revealed that TLR4 (L815A/L816A)-EGFP was not expressed on the plasma membrane regardless of whether MD-2 was cotransfected (Fig. 4*B*). The phenotype of this doubly substituted mutant appeared to be the same as that of the truncation mutants. These results imply that the leucines in positions 815 and 816 play an important role in TLR4 plasma membrane localization.

Analysis of Single Amino Acid Substitution Mutants—As previously mentioned, the amino acid sequence leucine-leucine at positions 815 and 816 does not completely match the dileucine motif, i.e. it lacks a preceding acidic amino acid. Therefore it

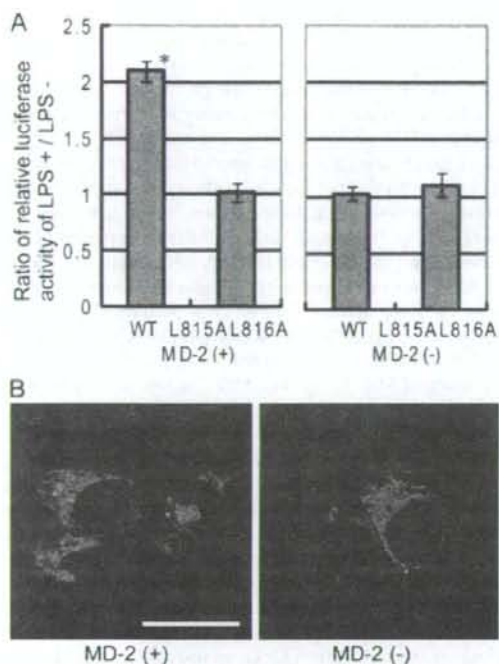


FIGURE 4. Leucines at positions 815–816 of TLR4 are responsible for impairment of LPS responsiveness and plasma membrane expression. **A**, the LPS stimulation assay was done for TLR4 (L815AL816A)-EGFP as in Fig. 2. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (*p* value for * are: TLR4 (WT)-EGFP/MD-2 (+), *p* = 0.002). **B**, TLR4 (L815AL816A)-EGFP expression in HEK293T cells was observed by laser confocal microscopy. Bar, 20 μ m.



FIGURE 5. Alignment of the cytoplasmic domain of EGFP fusion TLR4 amino acid-replacement mutants used in this study. TLR4 (L813A) signifies a mutant with leucine replaced with alanine at position 813. Others are named in the same manner. The amino acids are colored as in Fig. 1. All amino acids are designated using the single-letter code.

An Important Amino Acid of TLR4 for Its Function

was reasonable to explore whether leucines 815 and 816 need to be adjacent to each other. We created five genotypes of single amino acid mutants of TLR4: TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP. We excluded the amino acid at position 814 from the analysis, because the amino acid in position 814 of wild-type TLR4 is alanine. The amino acid sequence alignment of wild-type TLR4 and the single amino acid replacement mutants is shown in Fig. 5. DNA sequences were confirmed by sequencing.

As was done with truncation mutants, we measured NF- κ B activity of wild-type TLR4-EGFP, TLR4 (K813A)-EGFP, TLR4 (L815A)-EGFP, TLR4 (L816A)-EGFP, and TLR4 (D817A)-EGFP in response to LPS stimulation. All mutants except TLR4 (L815A)-EGFP showed responsiveness to LPS stimulation with coexpression of MD-2 (Fig. 6A). Without MD-2, no genotype of TLR4-EGFP responded to LPS stimulation (Fig. 6B). LPS stimulation was performed in an identical manner as with truncation mutants.

We analyzed the subcellular distribution of single amino acid mutants of TLR4-EGFP with and without MD-2 coexpression by fluorescence microscopy. TLR4 (K813A)-EGFP and TLR4 (D817A)-EGFP showed a similar fluorescence pattern to the wild-type, which localized at the plasma membrane when coexpressed with MD-2. No genotypes of TLR4-EGFP localized on the plasma membrane without MD-2 (Fig. 7). The cells transfected with TLR4 (L815A)-EGFP coexpressed with MD-2 did not show plasma membrane fluorescent pattern. Also, TLR4 (L815A)-EGFP showed comparatively weaker fluorescence than other mutants, possibly due to lower expression of the protein. Fluorescence of TLR4 (L816A)-EGFP with MD-2 was ambiguous as for the plasma membrane expression. Some kind of membranous structure was observed in the cytoplasmic area, but the intensity of the plasma membrane green fluorescence

was obscure. Together with the results from the LPS stimulation experiment, the leucines at positions 815 and 816 are considered to play important roles in signal transduction and/or subcellular distribution of TLR4.

Because EGFP consists of 239 amino acids, which is about one-third the size of the complete TLR4 protein, the experimental results obtained using TLR4-EGFP could have been influenced by the presence of the EGFP fused at the C terminus of TLR4. To rule out this possibility, we tested the functional integrity of both TLR4 (L815A) and TLR4 (L816A) with and without EGFP at the C terminus. Reporter assays were performed under the same conditions except that the shorter tag, FLAG-His₆, which has only 21-amino acid tags at the C terminus, was fused to TLR4 in place of EGFP. There was no difference

An Important Amino Acid of TLR4 for Its Function

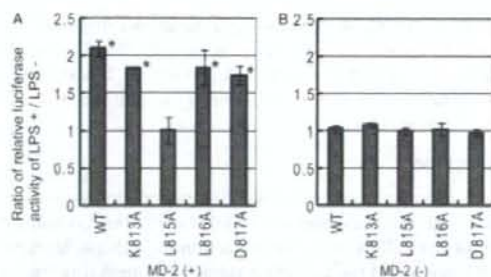


FIGURE 6. Leucine at position 815 of TLR4 is pivotal for LPS responsiveness as measured by NF- κ B luciferase assay. A, HEK293T cells were transfected with single amino acid replacement mutants of the human TLR4-EGFP fusion protein plasmid, human MD-2 plasmid, and luciferase reporter and control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. B, instead of MD-2, an empty vector was cotransfected with TLR4-EGFP plasmid and reporter assay vectors. LPS stimulation was done as in A. All results were expressed in the ratio of relative luciferase activity with LPS stimulation to that without the stimulation as in Fig. 2. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT)-EGFP/MD-2 (+), $p = 0.002$; TLR4 (K813A)-EGFP/MD-2 (+), $p = 0.000$; TLR4 (L816A)-EGFP/MD-2 (+), $p = 0.018$; and TLR4 (D817A)-EGFP/MD-2 (+), $p = 0.007$).

between EGFP-tagged proteins and FLAG-His₆-tagged proteins in the relative pattern of responsiveness against LPS stimulation (Fig. 8A). Because CD14 is also important for LPS recognition by TLR4, we examined the effect of CD14 coexpression on the phenotypic changes of the mutants (17, 18). Coexpression of CD14 did not change the phenotypes of wild-type TLR4, TLR4 (L815A), and TLR4 (L816A) in terms of LPS responsiveness (data not shown).

Cell surface expressions of the wild-type, L815A mutant, and L816A mutant TLR4-FLAG-His₆ fusion proteins were also examined. Live cells transfected with wild-type TLR4, the L815A mutant or the L816A mutant as well as human MD-2 and CD14 were biotinylated on the cell surface, and the biotinylated proteins were affinity-purified and subjected to Western blotting. Fig. 8B shows the marked difference in cell surface expression of wild-type and mutants L815A and L816A. Note that biotinylated proteins have additional residues on every amine of the extracellular domain, which leads to a band shift during electrophoresis. Although both mutants were detected far less than the wild-type on the cell surface, comparatively more L816A mutant was expressed on the plasma membrane than L815A mutant, and the amount of L815A mutant seemed to be negligible compared with the wild type. These results may clarify the ambiguity of the microscopic observation of TLR4 (L815A) and TLR4 (L816A). Plasma membrane expression of TLR4 was impaired when the leucine at 815 or 816 was replaced to alanine. But the leucine at 815 is more critical, and the mutant L816A may show the weaker phenotypic change.

To further investigate the characteristics of the TLR4 (L815A) mutant, we performed an immunoprecipitation assay of wild-type and mutant TLR4. Cells were transfected with a human MD-2-FLAG-His₆ expression vector and either the wild-type or the mutant (L815A) TLR4-EGFP expression vector. Anti-TLR4 monoclonal antibody (clone HTA125), anti-GFP polyclonal antibody, or anti-FLAG monoclonal antibody

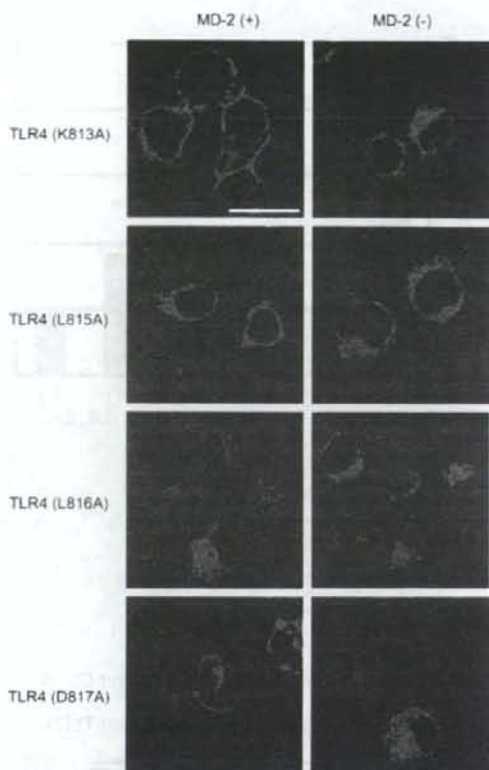


FIGURE 7. Leucines at the position 815 and 816 of TLR4 are responsible for full plasma membrane expression. Cells were cultured on coverslips in 12-well plates and transfected as in Fig. 2. EGFP-tagged TLR4 was visualized by laser confocal microscopy. Each genotype of TLR4-EGFP was cotransfected with human MD-2 plasmid or empty vector. Bar, 20 μ m.

was added to the lysate and precipitated with Protein G-Sepharose beads. Collected proteins were eluted and subjected to Western blotting. The results are shown in Fig. 8C. TLR4 (L815A) was not immunoprecipitated with anti-TLR4 antibody (HTA125). HTA125 antibody was raised against TLR4-expressing cells (9) and recognizes the extracellular portion of TLR4. This result suggests that the amino acid replacement at position 815 may cause a change in the extracellular portion of TLR4 and/or that the replacement may also inhibit cell surface expression of the mutant protein. On the other hand, both wild-type TLR4-EGFP and mutant TLR4-EGFP were immunoprecipitated with anti-GFP polyclonal antibody, which recognized EGFP. However, of the two bands of TLR4, the heavier band seems to be somewhat faint in the mutant, whereas in the wild type the heavier band is at least as dense as the lighter one. TLR4 can be detected as two separate bands in a Western blot (19), especially under transient transfection conditions. The difference in proportion of the heavy and light bands between wild-type and mutant TLR4 may suggest that there is some difference in glycosylation. Furthermore, wild-type TLR4 was coprecipitated with MD-2-FLAG-His₆, but the mutant TLR4 could not be detected (Fig. 8C, lanes 4 and 8). Because MD-2 is

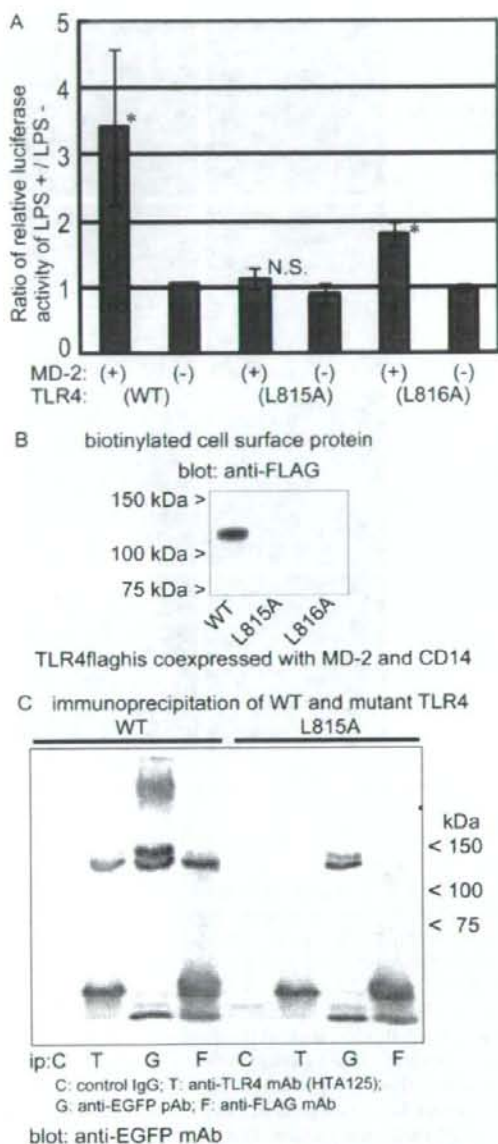


FIGURE 8. A, TLR4 mutants L815A and L816A with and without EGFP fusion exhibit the same phenotypes in LPS responsiveness and plasma membrane expression. HEK293T cells were transfected with the wild-type, the L815A or L816A mutant TLR4flaghis plasmid plus the human MD-2 plasmid and luciferase reporter, or control plasmids. After 36 h, cells were stimulated with LPS (10 ng/ml) for 7 h, and luciferase reporter gene activity was measured. The data were from three independent experiments. Small bars indicate 95% confidence intervals of the mean (p values for * are: TLR4 (WT) flaghis/MD-2 (+), $p = 0.046$; TLR4 (L816A) flaghis/MD-2 (+), $p = 0.003$). N.S.: not significant. B, wild-type and mutant TLR4s L815A and L816A were tagged by biotinylation of the cell surface proteins and affinity-purified. Human MD-2 and CD14 were coexpressed. TLR4 was visualized by immunoblotting using an anti-FLAG monoclonal antibody (mAb). Faint bands below 100 kDa are considered to be unbiotinylated intracellular TLR4 proteins that were not washed off during the process. Samples from TLR4 (WT), TLR4 (L815A), and TLR4 (L816A),

An Important Amino Acid of TLR4 for Its Function

associated with TLR4 (9), it is logical to expect that immunoprecipitating MD-2-FLAG-His₆ with anti-FLAG antibody should cause TLR4 to be coprecipitated with it. It is suggested by the result here that the association of the TLR4 mutant with MD-2 is impaired.

DISCUSSION

In this research, we performed mutagenesis analyses of particular amino acid residues in TLR4 to explore the mechanisms of TLR4 intracellular signal transduction and subcellular distribution. We found the candidate residues by analyzing truncation mutants of TLR4 in the cytoplasmic region, in which both signaling and normal subcellular distribution of TLR4 are disturbed. Because we are focusing on a common mechanism for the impaired signaling and distribution, we finally picked a single amino acid mutant that does not respond to LPS stimuli, as measured with NF- κ B reporter luciferase assay, and one that does not localize on the plasma membrane. TLR4 (L815A) is a mutant that meets these conditions, and our results suggest that the leucine at position 815 of TLR4 is required for both signal transduction and plasma membrane localization.

The best known single amino acid mutant of TLR4 is TLR4 (P712H) known as the *Lps^d* mutation in the C3H/He mouse, which corresponds to position 714 in this study of human TLR4 (5, 6, 20). Mice carrying this mutation opened up the rediscovery of TLR4 as a key player in innate immunity. Because this proline residue at this position is within the TIR domain and is conserved among TLRs or TLR4s of other species, it is assumed that the residue plays an important role in TLR4 function. The association of TLR4 (P712H) with its adapter proteins is reported to be intact, and the explanation for the functional impairment of TLR4 (P712H) is not clear (21–23).

Some single amino acid variants are found in humans, and these are related to the incidence or prognosis of some infections and other diseases. A growing body of data suggests that the ability of certain individuals to respond properly to TLR4 ligands may be impaired by single-nucleotide polymorphisms within TLR4 genes (24). The D299G and T399I alleles of the TLR4 gene have been associated with increased risk of severe infections (25).

By clarifying the subcellular component where the mutant protein is retained, or by clarifying to which compartment the mutant is not delivered, the abnormal intracellular sorting that is caused by the mutation in TLR4 (L815A) could be elucidated more precisely. Usually a sorting signal motif is comprised of several amino acids. In this regard, if the leucine at position 815 is a part of a motif, there should be other amino acids that are also members of the motif. Although replacement of leucine with alanine at position 816 did not cause an apparent signal transduction impediment, plasma membrane expression of TLR4 (L816A) was impaired to a certain extent. Positive

respectively, were prepared from the same number of cells as for the biotinylation experiment. C, immunoprecipitation with antibodies further reveals the characteristics of TLR4 (L815A). Anti-TLR4 monoclonal antibody (HTA125) does not precipitate the mutant TLR4, whereas anti-GFP polyclonal antibody (pAb) precipitates both wild-type and mutant TLR4. Mutant TLR4 was not coprecipitated with MD-2-FLAG-His₆. Lysates were prepared from cells transiently expressing wild-type or mutant TLR4-EGFP and MD-2-FLAG-His₆.

An Important Amino Acid of TLR4 for Its Function

response to LPS stimulation by TLR4(L816A) could be attributable to this small amount of expression on the plasma membrane. Mutagenesis analyses of neighboring amino acids of the leucine at 815 were not definitive, but the results could be suggestive that the adjacent leucine at 816 may work together with the leucine at 815. Leucines at position 815 and 816 could be in the same motif, and the leucine at position 816 may be less critical.

Several proteins have been reported to be involved in TLR4 cell surface expression. Heat shock protein gp96 is necessary for TLR4 association with MD-2 in the ER and for subsequent cell surface expression (26). PRAT4A and PRAT4B are associated with TLR4 and regulate TLR4 cell surface expression (27, 28). In embryonic fibroblasts of MD-2 knockout mice, TLR4 localization on the cell surface is severely impaired, and most TLR4 is retained in the ER or Golgi apparatus (15). MD-2 binds to TLR4 at its extracellular domain and is essential for LPS recognition by TLR4 (29). Although proteins such as CD14 and LPS-binding protein are reported to have important roles in LPS recognition by TLR4, in an *in vitro* setting HEK293T cells gain LPS responsiveness by introducing only TLR4 and MD-2 genes when measured by NF- κ B reporter assay (9, 30). Without transfection, HEK293 cells do not express TLR4, MD-2, or CD14, which are involved in LPS-induced intracellular signaling (31, 32). In this study, we show that the association of the TLR4 mutant and MD-2 is impaired (Fig. 8C).

Post-translational modification is another important factor for TLR4 function. Asparagine residues in the extracellular portion of TLR4 need to be glycosylated for plasma membrane expression of TLR4 (15, 19, 33). TLR4-MD-2 association is necessary for this glycosylation as well. The difference in the proportion of the heavy band to lighter band between wild-type and L815A mutant TLR4 immunoprecipitated with anti-GFP polyclonal antibody suggests that there may be some difference in glycosylation between wild-type and L815A mutant TLR4 (Fig. 8C). Although leucine at position 815 is located in the cytoplasmic tail of TLR4, we speculated that substitution of leucine at position 815 may cause a conformational change in the extracellular portion of the protein, which may interfere with the association between L815A mutant TLR4 and MD-2, leading to inhibition of glycosylation and cell surface expression of the mutant protein. Further investigation may reveal the mechanism involved in this phenotypic change in TLR4 (L815A), which would lead to better understanding of the mechanism of wild-type TLR4 signaling and trafficking.

Acknowledgment—We greatly appreciate the gift of human TLR4 and MD-2 cDNA from Dr. Kensuke Miyake (Institute of Medical Science, University of Tokyo, Japan).

REFERENCES

1. Takeda, K., Kaisho, T., and Akira, S. (2003) *Annu. Rev. Immunol.* **21**, 335–376
2. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabet, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., and Beutler, B. (2003) *Nature* **424**, 743–748
3. Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., and Seya, T. (2003) *J. Biol. Chem.* **278**, 49751–49762
4. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) *J. Immunol.* **169**, 6668–6672
5. Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) *J. Exp. Med.* **189**, 615–625
6. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282**, 2085–2088
7. Visintin, A., Mazzoni, A., Spitzer, J. A., and Segal, D. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 12156–12161
8. Nishitani, C., Mitsuzawa, H., Hyakushima, N., Sano, H., Matsushima, N., and Kuroki, Y. (2005) *Biochem. Biophys. Res. Commun.* **328**, 586–590
9. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189**, 1777–1782
10. Bonifacino, J. S., and Traub, L. M. (2003) *Annu. Rev. Biochem.* **72**, 395–447
11. Nishimura, N., and Balch, W. E. (1997) *Science* **277**, 556–558
12. Nufer, O., and Hauri, H. P. (2003) *Curr. Biol.* **13**, R391–393
13. Slack, J. L., Schooley, K., Bonner, T. P., Mitcham, J. L., Qvarnstrom, E. E., Sims, J. E., and Dower, S. K. (2000) *J. Biol. Chem.* **275**, 4670–4678
14. Latz, E., Visintin, A., Lien, E., Fitzgerald, K. A., Monks, B. G., Kurt-Jones, E. A., Golenbock, D. T., and Espevik, T. (2002) *J. Biol. Chem.* **277**, 47834–47843
15. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) *Nat. Immunol.* **3**, 667–672
16. Hein, C., and Andre, B. (1997) *Mol. Microbiol.* **24**, 607–616
17. Beutler, B. (2000) *Curr. Opin. Immunol.* **12**, 20–26
18. Akashi, S., Ogata, H., Kirikae, F., Kirikae, T., Kawasaki, K., Nishijima, M., Shimazu, R., Nagai, Y., Fukudome, K., Kimoto, M., and Miyake, K. (2000) *Biochem. Biophys. Res. Commun.* **268**, 172–177
19. Ohnishi, T., Muroi, M., and Tanamoto, K. (2003) *Clin. Diagn. Lab. Immunol.* **10**, 405–410
20. Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J. L., and Tong, L. (2000) *Nature* **408**, 111–115
21. Dunne, A., Ejdebäck, M., Ludidi, P. L., O'Neill, L. A., and Gay, N. J. (2003) *J. Biol. Chem.* **278**, 41443–41451
22. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O'Neill, L. A. (2001) *Nature* **413**, 78–83
23. Horng, T., Barton, G. M., and Medzhitov, R. (2001) *Nat. Immunol.* **2**, 835–841
24. Schroder, N. W., and Schumann, R. R. (2005) *Lancet Infect. Dis.* **5**, 156–164
25. Agnese, D. M., Calvano, J. E., Hahm, S. J., Coyle, S. M., Corbett, S. A., Calvano, S. E., and Lowry, S. F. (2002) *J. Infect. Dis.* **186**, 1522–1525
26. Randow, F., and Seed, B. (2001) *Nat. Cell Biol.* **3**, 891–896
27. Wakabayashi, Y., Kobayashi, M., Akashi-Takamura, S., Tanimura, N., Konno, K., Takahashi, K., Ishii, T., Mizutani, T., Iba, H., Kouro, T., Takaki, S., Takatsu, K., Oda, Y., Ishihama, Y., Saitoh, S., and Miyake, K. (2006) *J. Immunol.* **177**, 1772–1779
28. Konno, K., Wakabayashi, Y., Akashi-Takamura, S., Ishii, T., Kobayashi, M., Takahashi, K., Kusumoto, Y., Saitoh, S., Yoshizawa, Y., and Miyake, K. (2006) *Biochem. Biophys. Res. Commun.* **339**, 1076–1082
29. Nishitani, C., Mitsuzawa, H., Sano, H., Shimizu, T., Matsushima, N., and Kuroki, Y. (2006) *J. Biol. Chem.* **281**, 38322–38329
30. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., and Miyake, K. (2000) *J. Immunol.* **164**, 3471–3475
31. Espevik, T., Latz, E., Lien, E., Monks, B., and Golenbock, D. T. (2003) *Scand. J. Infect. Dis.* **35**, 660–664
32. Muta, T., and Takeshige, K. (2001) *Eur. J. Biochem.* **268**, 4580–4589
33. da Silva Correia, J., and Ulevitch, R. J. (2002) *J. Biol. Chem.* **277**, 1845–1854

Original Article

Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

Kazuhiko Koike,¹ Kuniyoshi Tsukada,¹ Hiroshi Yotsuyanagi,¹ Kyoji Moriya,¹ Yoshimi Kikuchi,² Shinichi Oka¹ and Satoshi Kimura²¹Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo and ²AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretroviral therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by dis-

tributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfecting with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfecting patients is expected.

Key words: coinfection, hepatitis C, HIV, liver disease

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.¹ The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.² Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.³ Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jirovecii*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have

declined. However, the reduction in mortality due to opportunistic infection has made patients coinfecting with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.^{4,5}

Coinfection with HIV has been shown to increase the HCV load in HCV infection,⁶ being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.^{7,8} It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.^{9,10} Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.¹¹ There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,¹² and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

Correspondence: Professor Kazuhiko Koike, Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kkoike-iky@umin.ac.jp

Received 7 August 2006; revision 22 August 2006; accepted 29 August 2006.

email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

METHODS

IN THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: http://www.acc.go.jp/mlhw/mlhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.¹² However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but

Table 1 Number of hospitals categorized by the number of patients infected with HIV and those coinfecting with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				Total
	0	1-19	20-49	50+	
0	43	52	5	1	101
1-9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

a majority of HIV-positive patients in Japan were enrolled in the study.

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20-49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfecting with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfecting patients in 18 (10.2%) hospitals. HIV/HCV coinfecting patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfecting patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfecting patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfecting patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission



Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (Δ) hospitals with 1-19 HIV-positive patients; (\square) hospitals with 20-49 HIV-positive patients; (\circ) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfected patients. Closed figures: hospitals with less than 10 HIV/HCV coinfected patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfected patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfected patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfected patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.¹² The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfected patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in

Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfected patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.¹³ In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.¹³ Therefore, an HCV prevalence of 4% in MSM

Table 2 Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfecting patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfecting patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfecting patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfecting with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

ACKNOWLEDGMENTS

WE THANK MS. Ogawa for her assistance in questionnaire inquiry. This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan. We thank the Hospitals in HIV/AIDS Network of Japan for the responses to the questionnaire, the list of which can be browsed at http://www.acc.go.jp/mlhw/mlhw_frame.htm.

REFERENCES

- Alter MJ. Hepatitis C virus infection in the United States. *J Hepatol* 1999; 31: 88-91.
- Weinbaum CM, Sabin KM, Santibanez SS. Hepatitis B, hepatitis C, and HIV in correctional populations: a review of epidemiology and prevention. *AIDS* 2005; 19: S41-6.
- Sulkowski MS. Hepatitis C virus infection in HIV-infected patients. *Curr HIV/AIDS Rep* 2004; 1: 128-35.
- Bica I, McGovern B, Dhar R *et al*. Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 32: 492-7.
- HCV in HIV. Challenges and opportunities. *The PRN NOTEBOOK* 2001; 6: 14-18. [Cited 15 July 2004.] Available from URL: <http://www.prn.org>.
- Picchio GR, Nakatsumo M, Boggiano C *et al*. Hepatitis C (HCV) genotype and viral titer distribution among Argentinian hemophilic patients in the presence or absence of human immunodeficiency virus (HIV) co-infection. *J Med Virol* 1997; 52: 219-25.
- Benhamou Y, Bochet M, Di Martino V *et al*. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfecting patients. *The Multivir Group Hepatology* 1999; 30: 1054-8.
- Marsen AH, Easterbrook PJ, Taylor C *et al*. Impact of human immunodeficiency virus (HIV) infection on the progression of liver fibrosis in hepatitis C virus infected patients. *Gut* 2003; 52: 1035-40.
- Staples CT Jr, Rimland D, Dudas D. Hepatitis C in the HIV (human immunodeficiency virus) Atlanta V.A. (Veterans Affairs Medical Center) Cohort Study (HAVACS): the effect of coinfection on survival. *Clin Infect Dis* 1999; 29: 150-4.
- Monga HK, Rodriguez-Barradas MC, Breaux K *et al*. Hepatitis C virus infection-related morbidity and mortality among patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 33: 240-7.
- Tatsunami S, Taki M, Shirahata A, Mimaya J, Yamada K. Increasing incidence of critical liver disease among causes of death in Japanese hemophiliacs with HIV-1. *Acta Haematol* 2004; 111: 181-4.
- National AIDS Survey Report. *The Ministry of Health, Welfare and Labor of Japan*. [Cited 10 July 2006.] Available from URL: <http://www.wam.go.jp/wamappl/bb14GS50.nsf/vAdmPBcategory40/73F664B9EA8D63D4492570290023D454?OpenDocument>.
- Tanaka J, Kumagai J, Katayama K *et al*. Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3 485 648 first-time blood donors during 1995-2000. *Intervirology* 2004; 47: 32-40.

Involvement of the PA28 γ -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein[∇]

Hironobu Miyamoto,¹ Kohji Moriishi,¹ Kyoji Moriya,² Shigeo Murata,³ Keiji Tanaka,³ Tetsuro Suzuki,⁴ Tatsuo Miyamura,⁴ Kazuhiko Koike,² and Yoshiharu Matsuura^{1*}

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,¹ Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo,² Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo,³ and Department of Virology II, National Institute of Infectious Diseases, Tokyo,⁴ Japan

Received 4 August 2006/Accepted 16 November 2006

The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 γ -dependent pathway. In this study, we examined whether PA28 γ is required for HCV core-induced insulin resistance *in vivo*. HCV core gene-transgenic mice lacking the PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$ CoreTg with PA28 γ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28 γ gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 γ -dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly in the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2

diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic β cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF- α) secretion (47) and that the HCV core protein is degraded through a PA28 γ /REG γ (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 γ enhances latent proteasome activity, although the biological significance of PA28 γ is largely unknown, with the exception that PA28 γ is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28 γ in the stability of IRS proteins and in the development of insulin resis-

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

[∇] Published ahead of print on 29 November 2006.

tance is not known. In this study, we examined the involvement of PA28 γ in the induction of insulin resistance by the HCV core protein *in vivo*.

MATERIALS AND METHODS

Preparation of PA28 γ -knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 $\gamma^{+/+}$ -CoreTg) line C49 and PA28 $\gamma^{-/-}$ mice have been described previously (35, 36). These two genotypes were crossed to create PA28 $\gamma^{+/+}$ -CoreTg mice. PA28 $\gamma^{-/-}$ -CoreTg mice were bred to generate PA28 $\gamma^{+/+}$ -CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 γ gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

Glucose tolerance test. The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

Insulin tolerance test. The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

Histological analysis of pancreatic islets. Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

Estimation of tumor necrosis factor alpha and HCV core protein. Mouse TNF- α was measured by using a mouse TNF- α ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

In vivo insulin stimulation and immunoblot analysis. Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28 γ , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na₂VO₄, 100 mM NaF, 50 mM Na₂P₂O₇, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate-2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF- α , IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5'-GGTACAACCCATCGGCTGGCA-3' (forward) and 5'-GCGACGTGGAAGTGGCAGAAG-3' (reverse) for TNF- α , 5'-ATAG

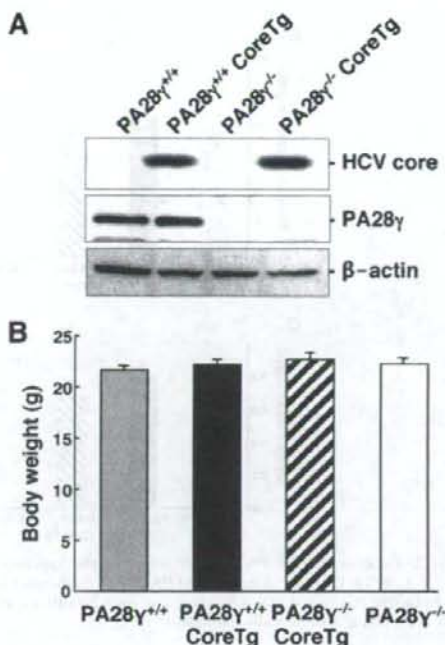


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 γ gene. (A) Expression of the HCV core protein and PA28 γ in the livers of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ -CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ -CoreTg mice. Lysates obtained from liver tissues of the mice (100 μ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 γ , and β -actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ($n = 7$ in each group). There were no statistically significant differences in body weights among the mice ($P > 0.05$).

CTCTGAGACCTTCTCAGCACCTAC-3' (forward) and 5'-GGAGITGCCCT CATTGTGCTAA-3' (reverse) for IRS1, 5'-AGCCTGGGGATAATGGT ACTATACCGA-3' (forward) and 5'-TTGTGGGCAAAGGATGGGGACAC T-3' (reverse) for IRS2, and 5'-CCAGCAAGCTTGCAACCTTAACCA-3' (forward) and 5'-GTAATGATCAGTCAACGGGGGAC-3' (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

Reporter assay for TNF- α promoter activity. The promoter region of the TNF- α gene (located from residues -1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglIII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf- α Pro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase (n -fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28 γ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

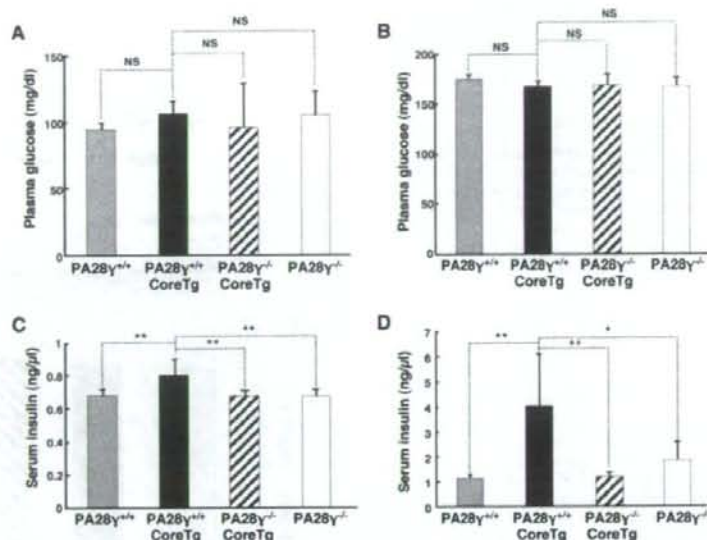


FIG. 2. Knockout of the PA28 γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice under fasting (A) or fed (B) conditions ($n = 7$ in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice ($n = 7$ in each group) are also shown. Values are represented as means \pm standard deviations. * $P < 0.05$; ** $P < 0.01$. NS, not statistically significant.

Statistical analysis. The results are presented as means \pm standard deviations. The significance of the differences was determined by Student's t test. P values of < 0.05 were considered statistically significant.

RESULTS

HCV core gene-transgenic mice deficient in the PA28 γ gene.

To investigate the role of PA28 γ in the development of insulin resistance in HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg)

mice, we generated HCV core gene-transgenic mice deficient in the PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg). A PA28 $\gamma^{+/+}$ CoreTg mouse expressing an amount of PA28 γ equal to that of its normal littermates (Fig. 1A) was crossed with a PA28 $\gamma^{-/-}$ mouse to generate a PA28 $\gamma^{+/+}$ CoreTg mouse. PA28 $\gamma^{+/+}$ CoreTg mice were bred with each other, and a PA28 $\gamma^{-/-}$ CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg

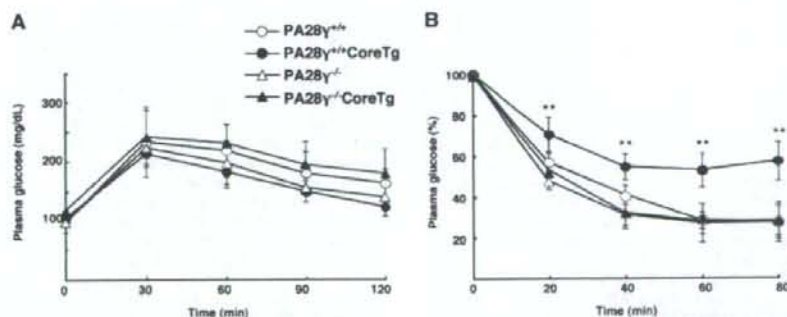


FIG. 3. Knockout of the PA28 γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ($n = 5$ in each group). There were no significant differences in glucose levels among the mice ($P > 0.05$). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration ($n = 5$ in each group). The values for the PA28 $\gamma^{+/+}$ (open circles), PA28 $\gamma^{+/+}$ CoreTg (closed circles), PA28 $\gamma^{-/-}$ (open triangles), and PA28 $\gamma^{-/-}$ CoreTg (closed triangles) mice are represented as means and \pm standard deviations. Significant differences in insulin sensitivity ($P < 0.01$) in PA28 $\gamma^{+/+}$ CoreTg mice compared to that in PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, or PA28 $\gamma^{-/-}$ CoreTg mice are indicated by double asterisks (**). There were no significant differences among PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice ($P > 0.05$).