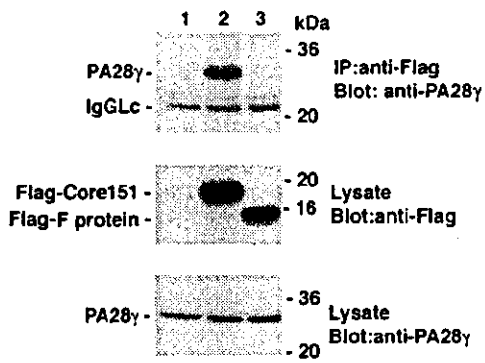

FIG. 9. The F protein of the −2/+1 frame does not bind to PA28γ. The plasmid encoding Flag-Core151 (lane 2) or Flag-F (lane 3) protein was transfected into 293T cells and harvested at 36 h posttransfection. Endogenous PA28γ was coimmunoprecipitated with anti-Flag antibody and then was visualized by immunoblotting with anti-PA28γ antiserum. The cells transfected with the empty plasmid were used as a negative control (lane 1). IP, immunoprecipitation; Lc, light chain.

Core151 but not with Flag-F protein (Fig. 9). These results suggest that the HCV core protein is processed by the cleavage of the C-terminal hydrophobic region and that the truncated core protein or the mature protein is translocated into the nucleus and degraded in a PA28γ-dependent manner.

DISCUSSION

The mechanism of hepatocellular carcinoma development in patients with chronic hepatitis C remains unclear. It has been demonstrated that expression of the HCV core protein alone is sufficient for the induction of hepatic steatosis and hepatocellular carcinoma in transgenic mice (28, 39, 41). These findings suggest that the HCV core protein plays a pivotal role in the development of hepatocellular carcinoma. In this study, we isolated PA28γ from a human fetal brain library as a host protein that specifically binds to the HCV core protein. We further suggest that HCV core protein interaction with PA28γ correlates with the retention of HCV core protein in the nucleus and regulates the stability of the HCV core protein in a proteasome-dependent manner. There are two isoforms of PA28γ in humans, a major form and a splicing variant that contains an additional 13 amino acids in the second helix domain. The second isoform is detected only in the human fetal brain and is not found in other human tissues or other mammals (3, 23). In this screen, we did not obtain the splicing variant of PA28γ from the human fetal brain library; it is, therefore, still unknown whether the human-specific isoform of PA28γ binds to the HCV core protein.

The C-terminal hydrophobic region of the HCV core protein is processed by host proteases such as signal peptidase and/or intramembrane proteases. The processed, mature HCV core protein transferred into lipid droplets when a full length of core protein was expressed by an alphavirus expression system (14, 27, 36). However, the mature core protein remained in the ER when the full length of core protein was expressed by transfection in this study (Fig. 3). This discrepancy might be due to the difference in expression systems, cell lines, and genotypes of the HCV clone.

When fused to EGFP, the PA28γ-binding region of the HCV core protein (EGFP-Core44-71) migrated into the nucleus, indicating that this region may function as an NLS. Deletion of the PA28γ-binding region from the HCV core protein (EGFP-Core151Δ44-71) or depletion of PA28γ from cells, however, did not eliminate nuclear transport of the HCV core protein, suggesting the presence of an alternative mechanism for the nuclear transport of the HCV core protein other than its interaction with PA28γ. Within the C-terminally truncated HCV core protein there exist three putative NLSs consisting of a cluster of basic amino acids (8, 55). β-Galactosidase-fused C-terminal truncated core protein lacking one of these clusters (β-gal-Core123Δ38-43) was localized primarily in the cytoplasm rather than the nucleus in COS cells (55); an EGFP-fused mutant, EGFP-Core151Δ38-43, however, was localized in the nucleus in the HeLa and 293T cell lines (data not shown). These results suggest that there are at least two possible mechanisms, PA28γ dependent and PA28γ independent, leading to nuclear transport of the HCV core protein. EGFP-Core151Δ38-43 and EGFP-Core151Δ44-71 are translocated into the nucleus by the PA28γ-dependent and -independent pathways, respectively. Both pathways may be mediated through importin or importin-like molecules because PA28γ has a c-Myc-like NLS in its homolog-specific region. Furthermore, the interaction with PA28γ was shown by time-lapse microscopy to play an important role in the retention of the HCV core protein in the nucleus. HCV core proteins lacking the PA28γ-binding region, EGFP-Core151Δ44-71 and EGFP-Core151, were exported from the nucleus to the cytoplasm in HeLa cells and embryonic fibroblasts derived from PA28γ knockout mice, respectively. The nuclear exporting signal was found in the C-terminal half of the HCV core protein and plays a role in the export of the HCV core protein from the nucleus to the cytoplasm (R. Suzuki, S. Sakamoto, T. Tsutsumi, A. Rikimaru, T. Shimoike, S. Machida, Y. Matsuura, T. Miyamura, and T. Suzuki, unpublished data). The putative PA28γ-dependent and -independent translocation of the HCV core protein from the cytoplasm to the nucleus, as well as the possible functions and fates of the HCV core protein in the nucleus, are illustrated in Fig. 10.

Although many host proteins have been reported to interact with the HCV core protein in relation to carcinogenesis (18, 33, 46, 66, 67), this is the first report demonstrating the interaction of the HCV core protein with an endogenously expressed host protein. In the livers of HCV core transgenic mice, the HCV core protein was primarily detected in the cytoplasm but some protein was found in the nucleus, albeit to a lesser extent (40). PA28γ was shown to coimmunoprecipitate with HCV core proteins irrespective of their intracellular localization (Fig. 2 and 3), suggesting that the core proteins bind to PA28γ after cell disruption. HCV core proteins truncated at the C terminus (HCV Core151 and 173) migrated into the nucleus and were degraded by ubiquitin-mediated proteolysis (57). In this study, overexpression of PA28γ led to the degradation of the HCV core protein; this degradation was able to be partially blocked by the proteasome inhibitor MG132. Additionally, HCV core protein was detected in the nucleus of a HeLa cell expressing the full-length HCV core protein in the presence of MG132 (Fig. 8). These results suggest that the

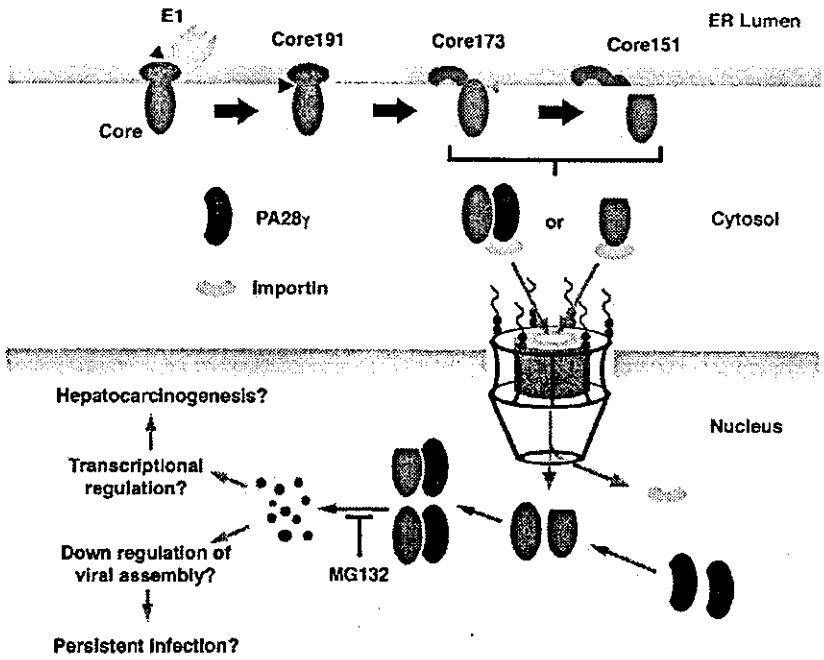

FIG. 10. Putative PA28γ-dependent and -independent translocation of the HCV core protein from the cytoplasm into the nucleus and possible function and fate. A precursor HCV core protein (Core191) is processed from a polyprotein in the ER by signal peptidase (red triangle), and then signal peptide peptidase (blue triangle) cleaves the C-terminal hydrophobic region, resulting in generation of mature core protein (Core173). The C-terminal region of the mature core protein is further processed by an unknown host protease (gray triangle) (Core151). Core173 and Core151 migrate into the nucleus through the nuclear pore complex, presumably by importin or an importin-PA28γcomplex. HCV core proteins interacting with PA28γ in the nucleus are degraded by the proteasome. This proteolysis is inhibited by the addition of the proteasome inhibitor MG132. Nuclear localization and degradation of the HCV core protein may induce down regulation of viral assembly, which may contribute to the maintenance of persistent infection with HCV. Furthermore,the resulting HCV core polypeptides might have some role as transcriptional regulators of host genomes, which are involved in hepatocarcinogenesis.

HCV core protein migrates into the nucleus and is then promptly degraded by the nuclear proteasome.

The F protein generated by ribosomal frameshift in the gene encoding the core protein was mainly localized in the cytoplasm and degraded by the proteasome (63). Although the expected mass of 14 kDa of the F protein from strain J1 was not detected in HeLa cells expressing HA-Core151 even in the presence of MG132 (Fig. 8B), we examined the interaction of the protein of −2/+1 frame of the gene encoding the HCV core protein with PA28γ. Lack of interaction of endogenous PA28γ with the F protein (Fig. 9) suggests that PA28γ specifically interacts with the HCV core protein but not with the F protein.

Hepatitis B virus X factor (HBx) alone induces hepatocellular carcinoma in mice (20, 24), suggesting that HBx plays an important role in hepatocellular carcinoma. HBx bound to PSMA7 and PSMC1, subunits of PA700 and the 20S proteasome, respectively, leads to the enhancement of the transcription activities of AP-1 and VP-16 (69). Like HBx, the HCV core protein is processed by the proteasome in a PA28γ-dependent manner. An HCV core protein with the same molecular mass as HCV Core151 was detected in cells in the presence of MG132 (57). The proteasome is well known to regulate many transcription factors such as NF-κB, p53, and c-Myc, etc. (4). For example, NF-κB and its inhibitor IκB are degraded by the proteasome, resulting in translocation of active NF-κB into the nucleus (19). Upon processing, the active form of NF-κB acquires transcription activity that regulates many biological functions such as cell proliferation (43). The HCV core protein is known as a regulatory factor that modulates some signaling pathways as well as affecting expression levels of a variety of proteins under the control of different promoters (reviewed in reference 56). The short-lived, C-terminally truncated HCV core protein may acquire an as yet undetermined biological function in the nucleus. Additionally, peptides derived from the HCV core protein that has been processed by the PA28γ-activated proteasome may play some role in the transcriptional regulation that is involved in hepatocellular carcinogenesis.

The PA28γ homopolymer is able to associate with the 20S proteasome (60) and strongly activates the peptidase activity of the latent proteasome (48). The PA28α/β heteropolymer forms a hybrid proteasome with the 20S proteasome and PA700; this complex efficiently enhances antigen processing in an ATP-dependent manner (59). The PA28γ homopolymer, PA700, and the 20S proteasome may also form a hybrid proteasome that may be responsible for the proteolysis of the HCV core protein in the nucleus. PA28γ knockout mice demonstrate no abnormality other than growth retardation; this suggests that PA28γ is either dispensable for host physiological function or that suitable compensation mechanisms exist within the organism (42). Translocation and degradation of the HCV core protein by the PA28γ-activated proteasome in the

nucleus may also contribute to the establishment and maintenance of persistent infection of HCV through the down regulation of viral assembly.

Although the biological significance of PA28γ is not well understood, in this study we have demonstrated new mechanisms by which PA28γ translocates and retains the HCV core protein in the nucleus; PA28γ is also involved in the proteolysis of the HCV core protein. Another nuclear proteasome activator, PA200, was recently purified from bovine testis and was demonstrated to enhance the peptidase activity but not the protease activity of the 20S proteasome (61). This report suggests that PA200 may be the functional homologue of PA28 in the nucleus. PA200 is predominantly localized to the nucleus and demonstrates homology to yeast and worm proteins that are implicated in the repair of DNA double-strand breaks. Thus, nuclear proteasome activity may be associated with DNA repair. Therefore, it may be possible that the interaction of PA28γ with the HCV core protein results in a perturbation of DNA repair activity through the nuclear proteasome, and these changes may subsequently induce hepatocellular carcinoma in humans and mice.

In conclusion, we have demonstrated that PA28γ specifically interacts with the HCV core protein in cell culture as well as in the livers of both HCV core transgenic mice and a patient with chronic hepatitis C. This interaction correlates to the nuclear retention and degradation of C-terminally truncated HCV core proteins. Understanding the precise function of PA28γ may give us new insight into virus-cell interactions and lead to a greater understanding of the pathogenicity of HCV infection. Establishment of HCV core transgenic mice deficient in PA28γ gene expression will allow the direct assessment of the involvement of PA28γ in the development of hepatocellular carcinoma induced by HCV core protein; these experiments are under way.

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REFERENCES

1. **Ahn, J. Y., N. Tanahashi, K. Akiyama, H. Hisamatsu, C. Noda, K. Tanaka, C. H. Chung, N. Shibmara, P. J. Willy, J. D. Mott, C. A. Slaughtere, and G. N. DeMartino.** 1995. Primary structures of two homologous subunits of PA28, a gamma-interferon-inducible protein activator of the 20S proteasome. FEBS Lett. 366:37–42.
2. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology 27: 621–627.
3. **Albertsen, H. M., S. A. Smith, S. Mazoyer, E. Fujimoto, J. Stevens, B. Williams, P. Rodriguez, C. S. Cropp, P. Slijepcevic, M. Carlson, M. Robertson, P. Bradley, E. Lawrence, T. Harrington, Z. Mei Sheng, R. Hoopes, N. Sternberg, A. Brothman, R. Callahan, B. A. J. Ponder, and R. White.** 1994. A physical map and candidate genes in the BRCA1 region on chromosome 17q12–21. Nat. Genet. 7:472–479.
4. **Almond, J. B., and G. M. Cohen.** 2002. The proteasome: a novel target for cancer chemotherapy. Leukemia 16:433–443.
5. **Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. 37:1802–1808.
6. **Brooks, P., G. Fuertes, R. Z. Murray, S. Bose, E. Knecht, M. C. Rechsteiner, K. B. Hendil, K. Tanaka, J. Dyson, and J. Rivett.** 2000. Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. Biochem. J. 346:155–161.
7. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA 91:8239–8243.
8. **Chang, S. C., J. H. Yen, H. Y. Kang, M. H. Jang, and M. F. Chang.** 1994. Nuclear localization signals in the core protein of hepatitis C virus. Biochem. Biophys. Res. Commun. 205:1284–1290.
9. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.
10. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88:2451–2455.
11. **Duttweiller, H. M.** 1996. A highly sensitive and non-lethal beta-galactosidase plate assay for yeast. Trends Genet. 12:340–341.
12. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. 67:2832–2843.
13. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–59.
14. **Hope, R. G., and J. McLauchlan.** 2000. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J. Gen. Virol. 81:1913–1925.
15. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61–68.
16. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology 14:381–388.
17. **Huang, D. C., S. Cory, and A. Strasser.** 1997. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. Oncogene 14:405–414.
18. **Jin, D. Y., H. L. Wang, Y. Zhou, A. C. Chun, K. V. Kibler, Y. D. Hou, H. Kung, and K. T. Jeang.** 2000. Hepatitis C virus core protein-induced loss of LZIP function correlates with cellular transformation. EMBO J. 19:729–740.
19. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. 18:621–663.
20. **Kim, C. M., K. Koike, I. Saito, T. Miyamura, and G. Jay.** 1991. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 351:317–320.
21. **Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, and R. H. Purcell.** 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675.
22. **Knowlton, J. R., S. C. Johnston, F. G. Whitby, C. Realini, Z. Zhang, M. Rechsteiner, and C. P. Hill.** 1997. Structure of the proteasome activator REGalpha (PA28alpha). Nature 390:639–643.
23. **Kohda, K., T. Ishibashi, N. Shimbara, K. Tanaka, Y. Matsuda, and M. Kasahara.** 1998. Characterization of the mouse PA28 activator complex gene family: complete organizations of the three member genes and a physical map of the approximately 150-kb region containing the alpha- and beta-subunit genes. J. Immunol. 160:4923–4935.
24. **Koike, K., K. Moriya, S. Iino, H. Yotsuyanagi, Y. Endo, T. Miyamura, and K. Kurokawa.** 1994. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology 19:810–819.
25. **Koike, K., T. Tsutsumi, H. Fujie, Y. Shintani, and M. Kyoji.** 2002. Molecular mechanism of viral hepatocarcinogenesis. Oncology 62(Suppl. 1):29–37.
26. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W. S. Lee, C. Kuo, K. Berger, F. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.
27. **Lemberg, M. K., and B. Martoglio.** 2002. Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Mol. Cell 10:735–744.
28. **Lerat, H., M. Honda, M. R. Beard, K. Loesch, J. Sun, Y. Yang, M. Okuda, R. Gosert, S. Y. Xiao, S. A. Weinman, and S. M. Lemon.** 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 122:352–365.
29. **Liu, Q., C. Tackney, R. A. Bhat, A. M. Prince, and P. Zhang.** 1997. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. J. Virol. 71:657–662.
30. **Lo, S. Y., F. Masiarz, S. B. Hwang, M. M. Lai, and J. H. Ou.** 1995. Differ-

ential subcellular localization of hepatitis C virus core gene products. Virology 213:455–461.
31. Lo, S. Y., M. Selby, M. Tong, and J. H. Ou. 1994. Comparative studies of the core gene products of two different hepatitis C virus isolates: two alternative forms determined by a single amino acid substitution. Virology 199:124–131.
32. Lo, S. Y., M. J. Selby, and J. H. Ou. 1996. Interaction between hepatitis C virus core protein and E1 envelope protein. J. Virol. 70:5177–5182.
33. Mamiya, N., and H. J. Worman. 1999. Hepatitis C virus core protein binds to a DEAD box RNA helicase. J. Biol. Chem. 274:15751–15756.
34. Masson, P., O. Andersson, U. M. Petersen, and P. Young. 2001. Identification and characterization of a Drosophila nuclear proteasome regulator. A homolog of human 11 S REGgamma (PA28gamma). J. Biol. Chem. 276: 1383–1390.
35. Matsuura, Y., T. Harada, M. Makimura, M. Sato, H. Aizaki, T. Suzuki, and T. Miyamura. 1994. Characterization of HCV structural proteins expressed in various animal cells. Intervirology 37:114–118.
36. McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. 21:3980–3988.
37. Moradpour, D., T. Wakita, K. Tokushige, R. I. Carlson, K. Krawczynski, and J. R. Wands. 1996. Characterization of three novel monoclonal antibodies against hepatitis C virus core protein. J. Med. Virol. 48:234–241.
38. Moriishi, K., D. C. Huang, S. Cory, and J. M. Adams. 1999. Bcl-2 family members do not inhibit apoptosis by binding the caspase activator Apaf-1. Proc. Natl. Acad. Sci. USA 96:9683–9688.
39. Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4:1065–1067.
40. Moriya, K., H. Fujie, H. Yotsuyanagi, Y. Shintani, T. Tsutsumi, Y. Matsuura, T. Miyamura, S. Kimura, and K. Koike. 1997. Subcellular localization of hepatitis C virus structural proteins in the liver of transgenic mice. Jpn. J. Med. Sci. Biol. 50:169–177.
41. Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. 78:1527–1531.
42. Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y. Nabeshima, K. Tanaka, and T. Chiba. 1999. Growth retardation in mice lacking the proteasome activator PA28gamma. J. Biol. Chem. 274:38211–38215.
43. Murray, R. Z., and C. Norbury. 2000. Proteasome inhibitors as anti-cancer agents. Anticancer Drugs 11:407–417.
44. Nikaido, T., K. Shimada, M. Shibata, M. Hata, M. Sakamoto, Y. Takasaki, C. Sato, T. Takahashi, and Y. Nishida. 1990. Cloning and nucleotide sequence of cDNA for Ki antigen, a highly conserved nuclear protein detected with sera from patients with systemic lupus erythematosus. Clin. Exp. Immunol. 79:209–214.
45. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.
46. Otsuka, M., N. Kato, K. Lan, H. Yoshida, J. Kato, T. Goto, Y. Shiratori, and M. Omata. 2000. Hepatitis C virus core protein enhances p53 function through augmentation of DNA binding affinity and transcriptional ability. J. Biol. Chem. 275:34122–34130.
47. Paesen, G. C., and P. A. Nuttall. 1996. A tick homologue of the human Ki nuclear autoantigen. Biochim. Biophys. Acta 1309:9–13.
48. Realini, C., C. C. Jensen, Z. Zhang, S. C. Johnston, J. R. Knowlton, C. P. Hill, and M. Rechsteiner. 1997. Characterization of recombinant REGalpha, REGbeta, and REGgamma proteasome activators. J. Biol. Chem. 272:25483–25492.
49. Rechsteiner, M., C. Realini, and V. Ustrell. 2000. The proteasome activator 11 S REG (PA28) and class I antigen presentation. Biochem. J. 345:1–15.
50. Roehrig, J. T., L. A. Staudinger, A. R. Hunt, J. H. Mathews, and C. D. Blair. 2001. Antibody prophylaxis and therapy for flavivirus encephalitis infections. Ann. N. Y. Acad. Sci. 951:286–297.
51. Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q. Choo, M. Houghton, and G. Kuo. 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87:6547–6549.
52. Santolini, E., G. Migliaccio, and N. La Monica. 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J. Virol. 68: 3631–3641.
53. Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura. 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J. Virol. 73:9718–9725.
54. Shoji, I., T. Suzuki, M. Sato, H. Aizaki, T. Chiba, Y. Matsuura, and T. Miyamura. 1999. Internal processing of hepatitis C virus NS3 protein. Virology 254:315–323.
55. Suzuki, R., Y. Matsuura, T. Suzuki, A. Ando, J. Chiba, S. Harada, I. Saito, and T. Miyamura. 1995. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. J. Gen. Virol. 76:53–61.
56. Suzuki, R., T. Suzuki, K. Ishii, Y. Matsuura, and T. Miyamura. 1999. Processing and functions of hepatitis C virus proteins. Intervirology 42:145–152.
57. Suzuki, R., K. Tamura, J. Li, K. Ishii, Y. Matsuura, T. Miyamura, and T. Suzuki. 2001. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 280:301–309.
58. Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. 65:1105–1113.
59. Tanahashi, N., Y. Murakami, Y. Minami, N. Shimbara, K. B. Hendil, and K. Tanaka. 2000. Hybrid proteasomes. Induction by interferon-gamma and contribution to ATP-dependent proteolysis. J. Biol. Chem. 275:14336–14345.
60. Tanahashi, N., K. Yokota, J. Y. Ahn, C. H. Chung, T. Fujiwara, E. Takahashi, G. N. DeMartino, C. A. Slaughter, T. Toyonaga, K. Yamamura, N. Shimbara, and K. Tanaka. 1997. Molecular properties of the proteasome activator PA28 family proteins and gamma-interferon regulation. Genes Cells 2:195–211.
61. Ustrell, V., L. Hoffman, G. Pratt, and M. Rechsteiner. 2002. PA200, a nuclear proteasome activator involved in DNA repair. EMBO J. 21:3516–3525.
62. Wilk, S., W. E. Chen, and R. P. Magnusson. 2000. Properties of the nuclear proteasome activator PA28gamma (REGgamma). Arch. Biochem. Biophys. 383:265–271.
63. Xu, Z., J. Choi, W. Lu, and J. H. Ou. 2003. Hepatitis C virus F protein is a short-lived protein associated with the endoplasmic reticulum. J. Virol. 77: 1578–1583.
64. Xu, Z., J. Choi, T. S. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. J. Selby, and J. Ou. 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. EMBO J. 20:3840–3848.
65. Yasui, K., T. Wakita, K. Tsukiyama-Kohara, S. I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J. R. Wands, and M. Kohara. 1998. The native form and maturation process of hepatitis C virus core protein. J. Virol. 72:6048–6055.
66. Yoshida, T., T. Hanada, T. Tokuhisa, K. Kosai, M. Sata, M. Kohara, and A. Yoshimura. 2002. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. J. Exp. Med. 196:641–653.
67. You, L. R., C. M. Chen, T. S. Yeh, T. Y. Tsai, R. T. Mai, C. H. Lin, and Y. H. Lee. 1999. Hepatitis C virus core protein interacts with cellular putative RNA helicase. J. Virol. 73:2841–28453.
68. Zhang, J., O. Yamada, H. Yoshida, T. Iwai, and H. Araki. 2002. Autogenous translational inhibition of core protein: implication for switch from translation to RNA replication in hepatitis C virus. Virology 293:141–150.
69. Zhang, Z., N. Torii, A. Furusaka, N. Malayaman, Z. Hu, and T. J. Liang. 2000. Structural and functional characterization of interaction between hepatitis B virus X protein and the proteasome complex. J. Biol. Chem. 275: 15157–15165.

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Antiapoptotic regulation by hepatitis C virus core protein through up-regulation of inhibitor of caspase-activated DNase

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Abstract

The hepatitis C virus (HCV) core protein is considered to influence multiple cellular processes. We developed a human hepatoblastoma HepG2-derived inducible cell line, Hep191, which allows tightly regulated expression of the core protein at relatively low but physiological levels under control of the ecdysone-regulated promoter. By transcriptional profiling, we identified differentially expressed genes, some of which are involved in cell growth or apoptosis such as inhibitor of caspase-activated DNase (ICAD), defender against cell death 1, tumor necrosis factor (TNF) receptor 1, and cytochrome c oxidase subunit VIII. Furthermore, we found that core protein expression increases a steady-state level of ICAD protein, possibly through enhancing its promoter activity, and inhibits caspase-3 activity induced by anti-Fas antibody. Since Fas- or TNF-mediated DNA fragmentation is suppressed in the core-induced Hep191 cells, these findings suggest that expression of HCV core at physiological levels confers blocking activity of caspase-activated DNase and consequently inhibiting apoptotic cell death.

Keywords: Hepatitis C virus; Microarray; DNA fragmentation; Apoptosis; Caspase-3

Introduction

A main characteristic feature of hepatitis C virus (HCV) infection is its persistent nature, which often leads to chronic hepatitis and liver cirrhosis. HCV infection is also strongly associated with the development of hepatocellular carcinoma (HCC) (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). Another characteristic is its highly specific host range for replication. To understand the exact mechanism of pathogenesis and persistence of HCV infection, it is important to elucidate virus–cell interaction, particularly in hepatic cells. We have been interested in the role of HCV core protein, which constitutes not only viral nucleocapsids but has multifunctions for pathogenesis or establishment of persistent infection of HCV (Lai and Ware, 1999; Thomson and Liang, 2000; Suzuki et al., 1999).

Apoptosis is definitely one of the key factors associated with liver injury and chronicity of HCV infection (Hayashi and Mita, 1997; Lau et al., 1998; Patel et al., 1998). However, its molecular mechanism is not clear yet. Apoptosis mediated by Fas or tumor necrosis factor (TNF) is a major pathway involved in a wide range of liver diseases. When

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Fas is constitutively expressed in hepatocytes and when agonistic anti-Fas antibodies are injected, the animals develop fatal fulminant hepatitis (Ogasawara et al., 1993; Lacronique et al., 1996). On the other hand, several HCV proteins have been reported to modulate apoptosis induced by diverse stimuli in different experimental systems (Ray et al., 1996, 1998; Fujita et al., 1996; Ruggieri et al., 1997; Zhu et al., 1998, 2001; Marusawa et al., 1999; Dumoulin et al., 1999; Gale et al., 1999; Hahn et al., 2000; Tai et al., 2000; Honda et al., 2000; Otsuka et al., 2002). However, the data shown to date, particularly with regards to the effects of the core protein, are not consistent. Several studies showed that the viral protein sensitizes apoptosis mediated by Fas, TNF-α, or other signaling pathways, whereas other studies showed inhibition of such apoptosis. Difficulty in unraveling of functions of HCV proteins on pathogenesis might be due to, at least in part, the fact that many previous investigations were based on transient or stable expression systems using unrelevantly high expression systems. In these systems, only certain populations of cells expressing viral proteins at high levels were selected. Such settings may be different from those of chronic infection, where only low production of viral proteins is observed.

In the current study, we overcame this problem through the use of the ecdysone-based inducible system, in which expression of HCV core protein can be induced at physiological levels in the same cellular background. The combination of transcriptional profiling with functional studies demonstrated the alteration of expression of several cellular genes including those related to apoptosis. It is of interest to note that expression of the core protein increased a steady-state protein level of inhibitor of caspase-activated DNase (ICAD), possibly through enhancing its promoter activity, and inhibited caspase-3 activity induced by anti-Fas antibody. In addition, induced expression of HCV core protein inhibited Fas- and TNF-mediated DNA fragmentation. These findings suggest that up-regulation of ICAD and inhibition of caspase-3 activity by the core protein collaboratively play roles in inhibiting apoptotic cell death through blocking activity of caspase-activated DNase (CAD).

Results

Establishment of a cell line allowing the tightly regulated expression of HCV core protein

The ecdysone-inducible system was used to generate tightly regulated cell lines expressing HCV core protein. This system is based on the binding of the steroid hormone ecdysone analog such as ponasterone A (PNA) to a heterodimeric receptor comprising a modified ecdysone receptor (VgEcR) and retinoid X receptor (RXR). The binding subsequently activates the ecdysone-responsive promoter to express the target genes (No et al., 1996). After keeping the transfected culture for 3 weeks in the presence of Hygromycin and Zeocin, 27 Zeocin-Hygromycin-resistant clones were isolated. They were then screened for regulated HCV core expression by sensitive fluorescence enzyme immunoassay (FEIA) and Western blot analysis. Being induced by treatment with PNA (0.3–30 μM) for 36 h, three of these clones (designated as Hep191) showed tightly regulated expression of the core protein. Western blot analysis of Hep191, clone 3 showed induction of 21-kDa HCV core protein in the presence of 30 μM PNA. It was not detected in the absence of PNA (Fig. 1A). FEIA analysis revealed that HCV core protein was observed as early as 3 h after addition of PNA and increased consistently up to 24 h. Continuous treatment with PNA showed that intracellular level of the core protein achieved after 24-h treatment was maintained during the whole culture period. Induced expression of the core protein was confirmed by indirect immunofluorescence staining (Fig. 1B). HCV core protein induced by treatment with 30 μM PNA for 48 h was localized exclusively within the cytoplasm. It seems that the core protein was essentially expressed uniformly in the induced Hep191 cells. No immunoreactivity was observed when the cells were cultured in the absence of PNA (data not shown).

We then examined "turning off" the regulation. Transcription intermediates of HCV core gene were monitored by reverse transcription-polymerase chain reaction (RT-PCR) in the cells in which the PNA medium was replaced by the vehicle-containing medium (Fig. 1C). No HCV cDNA transcript was detectable after 24 h of PNA removal. Correspondingly, HCV core protein level decreased and it was not observed after 24 h of withdrawal by Western blotting (data not shown). Further quantitative analysis by FEIA indicated that PNA's HCV core protein induction is in a concentration-dependent manner with the range of 0.3–30 μM of PNA for 36 h (Fig. 1D). No detectable level of HCV core protein was observed in the cells not treated with PNA, indicating negligible background of expression in the uninduced states. Notably, this core protein level in Hep191 is comparable to those in the livers of hepatitis C patients (6–1000 pg/mg total protein) and the transgenic mice developing hepatic steatosis and HCC (13–804 pg/mg total protein)(Koike et al., 2002; Tsutsumi et al., 2003).

Previous studies examining functional properties of HCV proteins have been performed mainly in cells overproducing the viral proteins. Moreover, many of them were nonhuman liver cells or human nonliver cells. Our human liver cell derived Hep191 cells allow the tightly regulated expression of core protein at a physiological level. This will provide a unique system to investigate possible roles of core protein in viral pathogenesis. In most of the following experiments, Hep191, clone 3 was used and core protein expression was induced by adding 10 μM PNA. Under this condition, the core protein level in the cells was approximately 500 to 1000 pg/mg total protein, which was little detected by Western blotting. We obtained similar results with the other two clones 21 and 37. These three clones

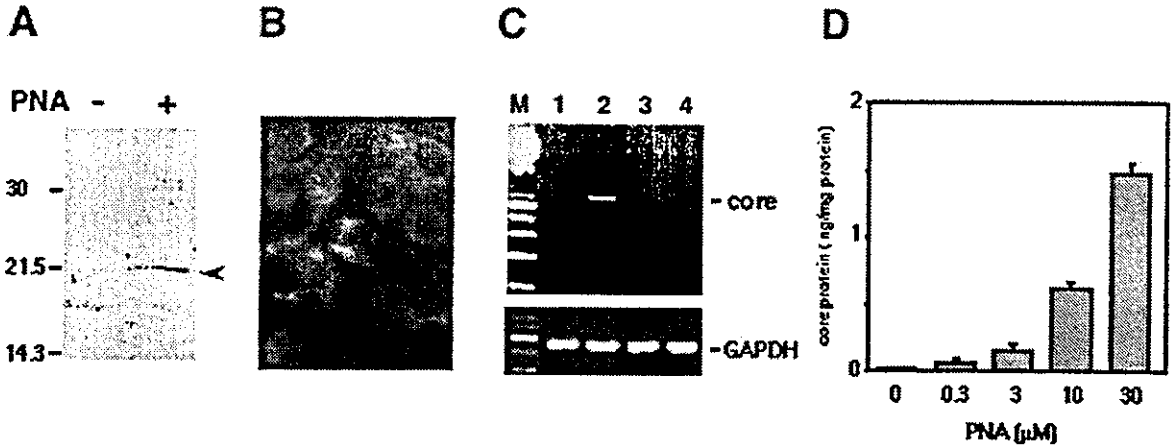

Fig. 1. Inducible expression of HCV core protein in Hep191 cells, clone 3. (A) Hep191 cells cultured in the presence (+) and absence (−) of 30 μM ponasterone A (PNA) for 36 h were analyzed by Western blotting with anticore monoclonal antibody. Arrow shows the core protein. The sizes in kDa of molecular mass markers are indicated on the left. (B) Indirect immunofluorescence staining of Hep191 cells after 48-h treatment with 30 μM PNA by monoclonal anticore antibody. (C) Tight control of the core gene expression was determined by RT-PCR. Total RNAs were isolated from Hep191 cells before PNA treatment (lane 1), after 24-h treatment (lane 2). Subsequently, the treated cells were washed, followed by another culture in the vehicle-containing medium for 24 h (lane 3) or 48 h (lane 4). RT-PCR was performed using primers specific for genes of the core (top) and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) (bottom). Molecular size marker is shown at the left sides in both panels (M). (D) Core protein levels in Hep191 cells cultured with various concentrations of PNA for 36 h was quantified by FEIA. Data shown represent the mean values with standard deviation (S.D.) of triplicate experiments.

have been maintained in culture for over 20 months and more than 90 passages without loss of tightly regulated gene expression.

Cell growth properties

To test the effects of induced expression of HCV core protein on cell growth, Hep191 cells were cultured in daily changed medium with or without PNA. The number of viable cells was determined by counting daily for 10 days. As shown in Fig. 2A, no differences were noted between growth curves of Hep191 cultured in the medium with 10% fetal bovine serum (FBS) in the presence and absence of PNA. Furthermore, the same saturation density was reached under both conditions. The result demonstrates that HCV core protein expressed in Hep191 cells has no influence on growth capacity, which is consistent with that in previous studies using human hepatoma Huh-7 cells (Li et al., 2002) and human osteosarcoma U-2OS cells (Moradpour et al., 1996) with tetracycline-inducible systems. Analogous results were found in slower growing Hep191 cells cultured in 1% FBS-containing medium (Fig. 2B), suggesting that core expression does not confer serum independence to the cells. We also observed that the inducing agent, PNA, exerted no pleiotropic effects, confirming the advantage of using ecdysteroids, which are known not to affect physiology of mammalian cells.

Changes in gene expression in the core-induced Hep191 cells

We then compared the gene expression patterns in Hep191 cells in the presence and absence of 10 μM PNA.

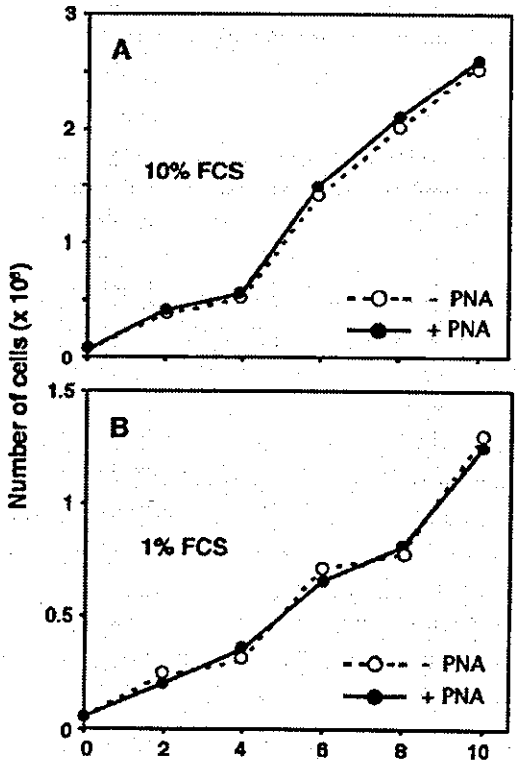

Fig. 2. Cell growth analysis. Hep191 cells were grown in the medium with 10% (A) and 1% (B) FBS in either the presence (closed circle) or the absence (open circle) of 10 μM PNA and were counted every other day as described under Materials and methods. Results show the mean cell numbers in each dish obtained from triplicate experiments.

Table 1
Genes up- or down-regulated by expression of HCV core protein in Hep191 cells

Accession no.	Gene name	Category	Fold change
AK091194	Probable trans-1,2-dihydrobenzene-1,2-dioldehydrogenase	Metabolism	2.3
Y12653	Diubiquitin	Immune response	2.2
M90657	Tumor-associated antigen L6	Tumor associated	2.2
Y12065	Nucleolar protein hNop56	RNA/protein metabolism	2.1
S54005	Thymosin beta-10	Cell motility	2.1
X74981	Keratin 8	Cell structure	2.1
M29064/M29065	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA/protein metabolism	2.1
U91985	Inhibitor of caspase-activated DNase (ICAD)	Apoptosis	2.1
AH000826	Dihydrodiol dehydrogenase	Metabolism	2.1
D15057	Deffender against cell death 1, DAD1	Apoptosis	2.0
L22009	hnRNP H	RNA/protein metabolism	2.0
P05215	Tubulin alpha-4 chain	Cell structure	2.0
AF517226	Insulin-like growth factor 2 (somatomedin A)	Signaling	0.3
X54989	Evi-1	Signaling	0.4
M55422	Krueppel-related zinc finger protein, H-plk	Transcription	0.4
J04823	Cytochrome C oxidase subunit VIII-liver/heart precursor	Apoptosis	0.4
P09565	Putative insulin-like growth factor II associated protein	Signaling	0.4
M75866	TNF receptor 1	Apoptosis	0.4
BC037225	AP-2 beta (activating enhancer-binding protein 2 beta)	Transcription	0.5
U10439	Double-stranded RNA adenosine deaminase	RNA/protein metabolism	0.5

Note. Genes with two-fold or more increase (top) or decrease (bottom) in average of two experiments are indicated.

A cDNA microarray consisting of 2304 human genes was used. cDNA labeling reactions were carried out in duplicate where the fluorescent dyes, Cy3 and Cy5, were switched during the synthesis, followed by hybridizing each probe pair to a separate microarray. A list of the genes demonstrating greater than two-fold change in average of two experiments is provided in Table 1. We observed 12 genes up-regulated and 8 genes down-regulated upon induction of the core protein. Most of the genes differentially expressed were regulators of either transcription, RNA/protein metabolism, apoptosis, or signaling molecules. Among these, four genes which are involved in apoptosis regulation are noteworthy: the expression of (i) ICAD and (ii) defender against cell death 1 (DAD1) was increased, whereas the expression of (iii) TNF receptor 1 (TNFR1) and (iv) cytochrome c oxidase subunit VIII was decreased in the core-induced Hep191 cells.

To validate the differential expression of these genes, we performed semiquantitative RT-PCR analysis. For each assay, the reaction conditions and cycle numbers of PCR were individually optimized and adjusted so that the reaction fell within the linear range of product amplification. Glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), whose expression was not altered, was used as a control for loading. Our RT-PCR qualitatively agreed with the expression patterns generated by microarray data (Fig. 3A). PNA itself had no effect, or little if any, on these gene expressions in control HepRXR cells, which are not expressing the core protein. In addition to analyzing clone 3 of Hep191, semiquantitative RT-PCR was also performed with ICAD and GAPDH genes of clone 37 (Fig. 3B). In good agreement with that observed with clone 3, expression of ICAD gene was up-regulated after induced expression of the core protein in clone 37.

Expression of the core protein resulted in activation of the ICAD promoter

We next focused on the impact of the core protein on ICAD expression. ICAD is a 45-kDa inhibitor of CAD, caspase-activated DNase, of 40-kDa (Liu et al., 1997; Enari et al., 1998). ICAD exists as a complex with CAD in

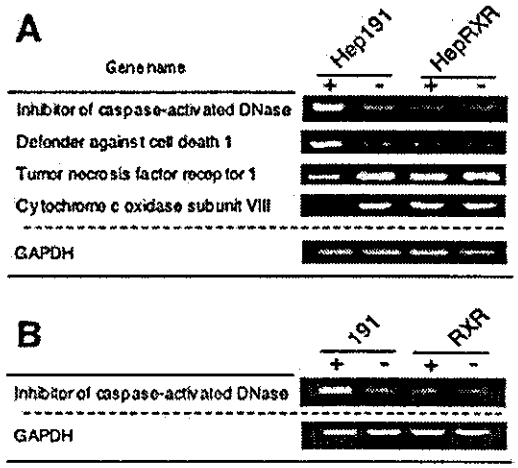

Fig. 3. Semiquantitative RT-PCR analysis of differentially expressed genes which are related to apoptosis. Total RNAs were extracted from Hep191 clone 3 (A) or clone 37 (B) and HepRXR cells cultured either with (+) or without (−) 10 μM PNA, followed by semiquantitative RT-PCR analysis. GAPDH was amplified in parallel as a control.

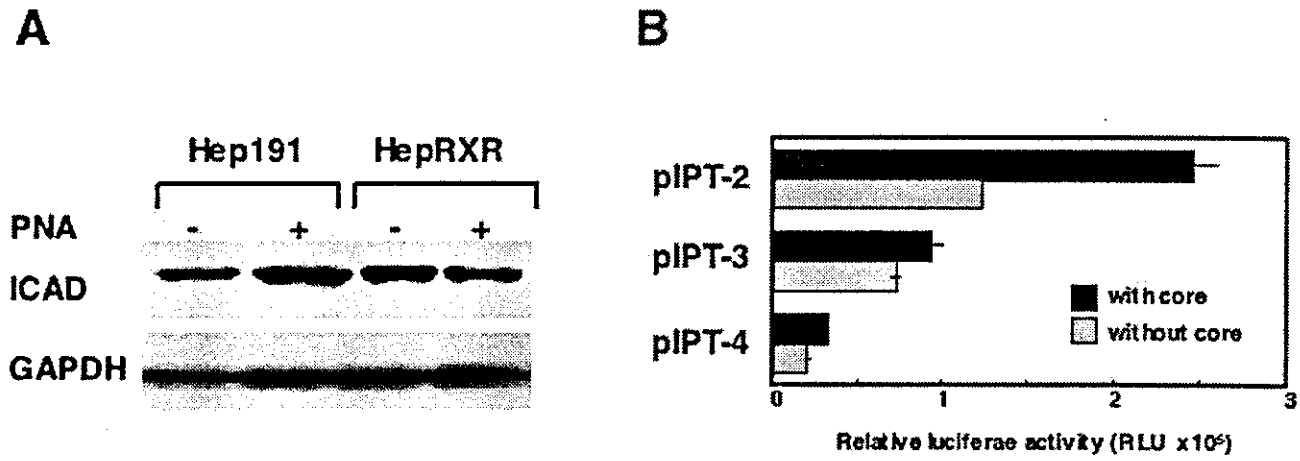

Fig. 4. Up-regulation of ICAD expression by the core protein. (A) ICAD and GAPDH expressed in Hep191 and HepRXR cells in the absence (−) or the presence (+) of PNA were analyzed by Western blotting. (B) 293T cells were cotransfected with the ICAD promoter/luciferase construct (either pIPT-2, -3, or -4) and the internal control pRL-TK in the presence of core-expressing pCAGC191 or control empty vector. Relative luciferase activity (RLU) was determined at 24 h posttransfection. Results are expressed as a mean ± S.D. of triplicate experiments.

proliferating cells. Upon apoptotic stimuli, caspases, particularly caspase-3, proteolytically process ICAD to release CAD from the complex, allowing CAD to catalyze the fragmentation of chromosomal DNA. As shown in Fig. 4A, we found a significant increase in ICAD protein in Hep191 cells treated with 10 μM PNA for 48 h by Western blotting, whereas no change was observed in the control cells with the same treatment.

To address the molecular mechanism of up-regulation of ICAD expression by core protein, we examined whether the core expression is linked to ICAD promoter activity. ICAD promoter/luciferase reporter constructs used in this study are generous gifts from Dr. S. Nagata (Kawane et al., 1999). However, HepG2-based Hep191 cells have low transfection efficiency, and these cells were not suitable for cotransfection experiments of control and experimental reporter vectors. We therefore adopted the 293T transient transfection system, in which transfection efficiency of each plasmid exhibits constantly about 80%. As shown in Fig. 4B, luciferase activity expressed from ICAD reporter construct, which includes the 110-bp 5′ flanking region of the ICAD gene (pIPT-2), increased twofold following expression of the core protein. On the other hand, the 39-bp deletion at 5′ region of the promoter (pIPT-3) resulted in a marked decrease with the core expression. The 39-bp region thus plays a role in regulating ICAD promoter activity by HCV core protein. Further 24-bp deletion (pIPT-4) caused threefold reduction of the activity in cells in both the presence and the absence of the core protein. The ICAD gene used in this reporter assay was murine in origin, but its 110-bp promoter region has 75% sequence identity to that in human ICAD gene. Furthermore, the putative binding sites for transcription factors, such as USF1 and GATA-1 in the 39-bp region, are well conserved between murine and human ICAD genes. Thus, these data indicate that stimulation of ICAD promoter activity by the core protein results in an elevated level of ICAD protein in the core-expressing cells.

Effects of HCV core protein expression on Fas- and TNF-α-mediated DNA fragmentation in Hep191 cells

The above observation showing changes in expression of several apoptosis-related genes prompted us to study the role of HCV core protein in Fas- or TNF-α-mediated apoptosis pathway. Hep191 cells with or without core induction were treated with anti-Fas antibody for 14 h. DNA ladders due to activation of nuclear endonucleases were then measured by quantitating cytoplasmic mono- and oligonucleosomes. As indicated in Fig. 5A, Hep191 cells treated with 10 μM PNA showed decreased sensitivity against anti-Fas-induced DNA fragmentation. DNA-associated mono- and oligonucleosomes released into cytoplasm were reduced to 28% in the core-expressing Hep191 cells. To exclude the direct effects of PNA in apoptotic response, we examined the Fas-induced DNA fragmentation also in the control HepRXR cells. No difference in apoptotic response was observed in HepRXR cells in either the presence or the absence of PNA (Fig. 5A). Prolonged induction of the core protein expression for 15 or 30 days with 10 μM PNA also inhibited the Fas- and TNF-α-mediated apoptosis (Fig. 5B).

We further investigated the relation of induced HCV core protein and Fas-mediated apoptotic response. As shown by the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) technique in Fig. 6, anti-Fas-treated Hep191 cells in the absence of PNA underwent apoptosis. Apoptotic cells showing DNA strand breaks were clearly demonstrated by in situ labeling. In contrast, the cells expressing HCV core protein exhibited little detectable fluorescence.

We further examined whether the induction of HCV core

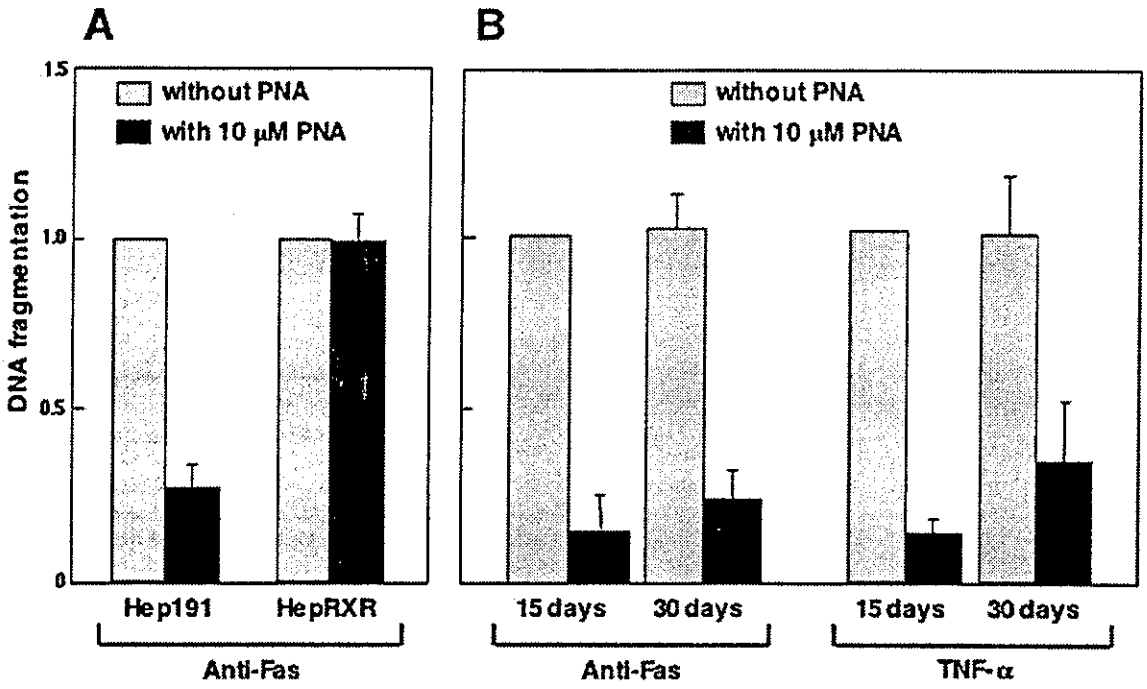

Fig. 5. Quantitation of apoptotic DNA fragmentation. (A) Hep191 and HepRXR cells, which cultured in the presence or absence of 10 μM PNA for 36 h, were treated with anti-Fas antibody and CHX for a further 14 h. Apoptotic DNA fragmentation was measured using a sandwich enzyme-linked immunosorbant assay system for cell death detection. Relative values are shown as means ± S.D. of independent three experiments. (B) Hep191 cells were grown in the presence of 10 μM PNA with repeated replacing the medium for 15 or 30 days, followed by treating with either anti-Fas/CHX for 14 h or TNF-α for 48 h.

protein would inhibit the anti-Fas-stimulated caspase-3 activity. Treatment of the core-induced Hep191 cells with anti-Fas resulted in a decrease in the caspase-3 (or DEVDase) activity in these cell lysates (Fig. 7A). Addition of PNA may not affect the enzyme activity per se, because no caspase-3 activity change was observed in HepRXR cells in the presence of 10 μM PNA. However, it is known that the treatment with anti-Fas antibody triggers the cleavage of the inactive proform of caspase-3 by an upstream caspase to generate its active subunits (Dubrez et al., 1996). Thus, we analyzed the level of 32-kDa procaspase-3, which decreases upon enzyme activation, by Western blotting (Fig. 7B). As expected, Hep191 cells without PNA treatment demonstrated a decrease in the amount of the procaspase-3 in response to anti-Fas antibody. By contrast, the level of procaspase-3 in the cells treated with 10 μM PNA was comparable to that under no apoptotic stimuli. We observed similar results in the other Hep191 clone, 37 (Fig. 7C), confirming that activation of caspase-3 following treatment with anti-Fas antibody was inhibited by induced expression of the core protein. Since caspase-3 locates almost at the end of the apoptotic cascade, its inhibition strongly indicates

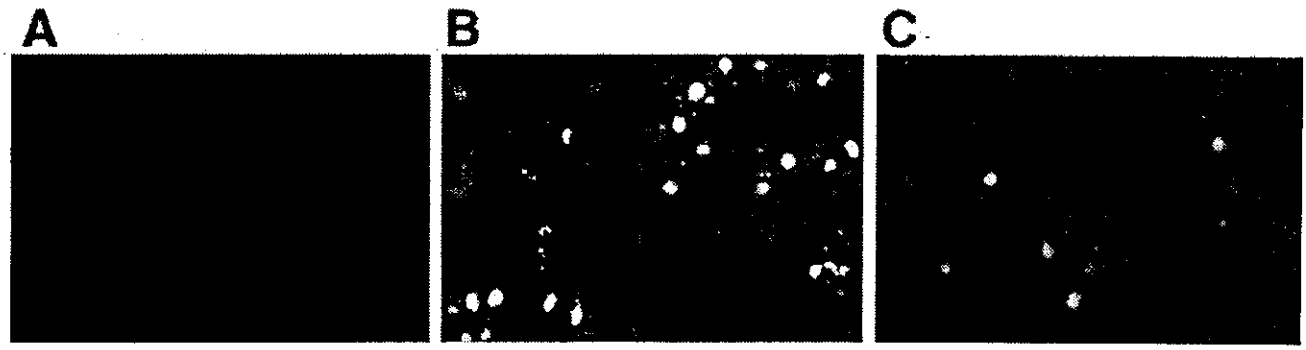

Fig. 6. Photomicrographs of Hep191 cells with induced expression of the core protein. Cells were cultured in the absence (B) or presence (C) of 10 μM PNA for 36 h, followed by treating with anti-Fas antibody and CHX for a further 14 h. PNA-treated cells without inducing apoptosis by anti-Fas/CHX were also prepared (A). Apoptotic cells were visualized by TUNEL fluorescence analysis.

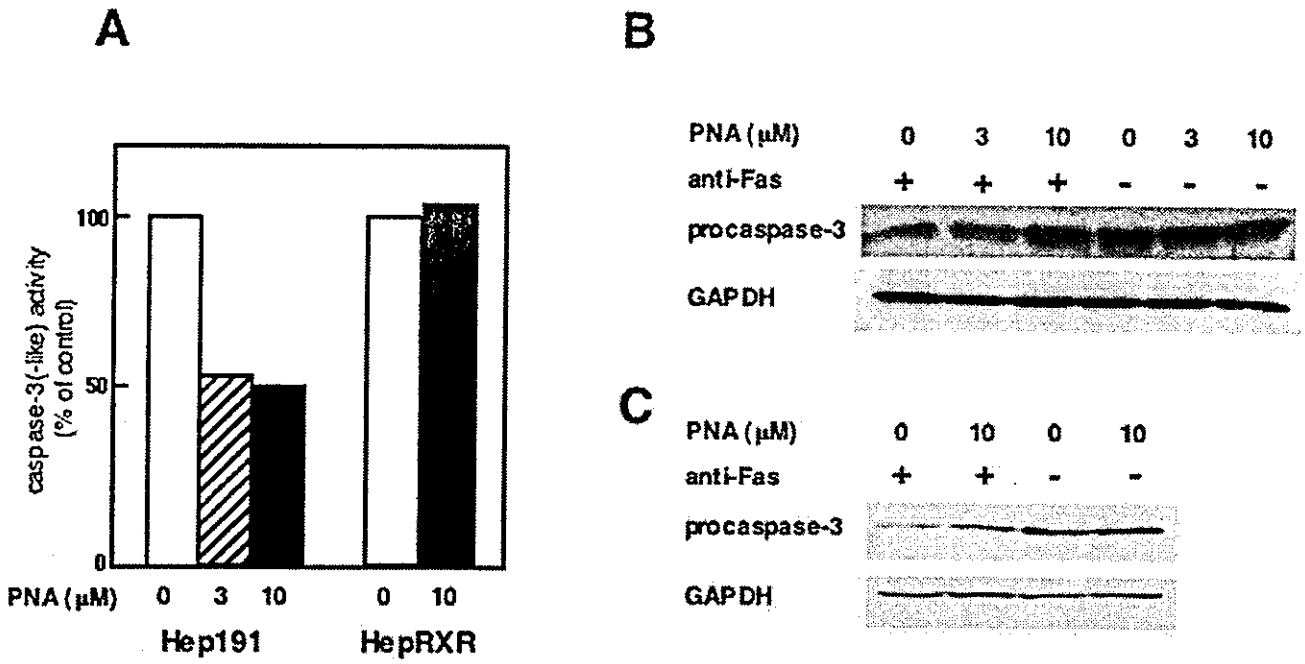

Fig. 7. Effect of the core protein on enzymatic activity and processing of caspase-3 (A) Hep191 (clone 3) and HepRXR cells grown in the presence of 0–10 μM PNA for 40 h were treated with anti-Fas/CHX, followed by measuring caspase-3 activity in cell lysates as described under Materials and methods. Results show the mean values of triplicate experiments. (B) Lysates from Hep191 cells (clone 3) cultured in the presence of 0–10 μM PNA with or without anti-Fas treatment were analyzed by Western blotting to detect procaspase-3 and GAPDH. (C) Same as in (B), but with the other Hep191 clone, 37.

that induced expression of the HCV core protein at moderate levels inhibits Fas-mediated DNA fragmentation in human liver cells.

Discussion

Deregulation of apoptosis or programmed cell death is involved in a wide range of pathological processes, including autoimmune hepatitis, fulminant hepatitis, and HCC. Although there is increasing evidence that the HCV core protein interacts with regulatory pathways controlling apoptosis, the role of this protein remains controversial because of the conflicting results in the literature, that is, its opposite effects in either inhibition or potentiation of apoptosis. It is likely that the expression level of the core protein, availability of apoptotic versus survival factors, and experimental setting might affect the fate of cells exposed to diverse stimuli.

In the current study, we have introduced the ecdysone-inducible expression system to establish a human hepatoblastoma HepG2-based stable cell line, Hep191, allowing tightly regulated expression of HCV core protein. In this system, no background expression is observed in the absence of the inducer. It is also possible to precisely control the expression levels in response to varying inducer concentrations. These are the advantages of using the ecdysone-based system over other inducible systems, such as the tetracycline-based system. Indeed, the ecdysone-inducible system was developed to address a main flaw of the tetracycline-inducible system, a relatively high background of expression in the uninduced state (No et al., 1996). In contrast to tetracycline, the lipophilic nature of the ecdysteroids such as PNA allows their efficient penetrance into cells and appropriate pharmacokinetics that prevent storage and expedite clearance, leading to a delicate temporal control of gene expression. Further, we observed no pleiotropic effects of the inducing agent. Neither cytotoxicity nor influence on cell proliferation was observed in PNA-treated cells (Fig. 2). The lack of baseline cross-talk between the inducible system and endogenous cellular pathway is essential to study HCV–liver cell interaction. It is also noteworthy that the expression levels of HCV core protein in Hep191 cells can be controlled to the level comparable to those in the livers of hepatitis C patients and the HCV core-transgenic mice developing hepatic steatosis and HCC (Koike et al., 2002; Tsutsumi et al., 2003). In this regard, most of the previous studies on possible roles of HCV core protein in viral pathogenesis depended on overexpression systems of either constitutively expressing cell lines or transiently transfected cells. Thus, Hep191 cells based on the ecdyson system may be useful as a model of HCV core–host cell interaction closer to the virus infection setting than previous expression systems. Liver cell specific interaction included in this system would be important.

We applied this Hep191 cell line to analyze the gene

expression profiling by using cDNA microarray technique. Clone-to-clone variability of cells can be controlled in this inducible system. Taking this advantage, we investigated the response of the same cells to two different stimuli. We identified 20 genes (0.9% of 2304 transcripts examined) which showed twofold or more difference between with and without the core induction. Among them were several genes related to transcriptional regulation, signaling, RNA/protein metabolism, and apoptosis. We subsequently performed semiquantitative RT-PCR of four apoptosis-related genes and confirmed that ICAD and DAD1 were up-regulated, and TNFR1 and cytochrome c oxidase subunit VIII were down-regulated in the core-expressing cells.

With regard to ICAD, its promoter activity was up-regulated by the core protein expression and steady-state level of ICAD protein was subsequently increased. ICAD, otherwise known as DFF45 (DNA fragmentation factor 45-kDa subunit), is a caspase-3 substrate which is cleaved before apoptotic DNA fragmentation proceeds. Moreover, ICAD is a specific inhibitor of CAD, also called DFF40 (DNA fragmentation factor 40-kDa subunit), and is a molecular chaperone which is involved in proper folding of the endonuclease (reviewed by Nagata, 2000). It has been reported that overexpression of ICAD inhibits staurosporine- or Fas-induced DNA fragmentation (Sakahira et al., 1998). To our knowledge, the data presented here are the first to demonstrate increased expression of ICAD mediated by a viral protein through enhancing its promoter activity. In this study, we observed that in the core-expressing Hep191 cells expression of CAD did not change, as determined by Western blotting as well as cDNA microarray (data not shown). The maintenance of a 1:1 ratio of ICAD and CAD might be important for the homeostasis of normal cells, because an excess of ICAD will increase the threshold for downstream apoptotic signaling. Thus, our findings suggest that up-regulation of ICAD by HCV core protein may contribute to metabolic perturbation such as inhibition of apoptosis in HCV-infected hepatocytes.

The DAD1 gene, identified as a defender against apoptotic death gene, was also up-regulated. The DAD1 gene encodes a subunit of the oligosaccharyltransferase enzyme complex that initiates N-linked glycosylation (Nakashima et al., 1993). This protein plays a role in inhibition of apoptosis in some cultured cells (Yoshimi et al., 2000). Studies with mice carrying a null allele for DAD1 showed that this gene is required for proper processing of N-linked glycoproteins and for certain cell survival (Hong et al., 2000). It is also of interest that DAD1 gene is highly expressed in HCC cells compared to adjacent nontumorous liver tissues through differential display analysis (Tanaka et al., 2001).

TNFR1 is one of death receptors and relays death signals through a cytoplasmic sequence motif called the death domain. Upon TNF engagement, TNFR1 binds other death domain-containing proteins and subsequently leads to activation of caspases, resulting in apoptosis (for review, see Ashkenazi and Dixit, 1998). Down-regulation of TNFR1 in our study is not consistent with the previous report showing HCV core protein binds to the cytoplasmic domain of TNFR1 and enhances TNF-induced apoptosis of certain mouse and human cells. In these cells, the presence of core protein did not alter the expression of TNFR1 (Zhu et al., 1998, 2001). The difference in the conditions of either cell culture or core expression may be attributed to this discrepancy.

Cytochrome c oxidase is the terminal enzyme complex in the mitochondrial respiratory chain, and cytochrome c oxidase subunit VIII is one of the 13 subunits of the complex. A progressive reduction in respiratory activity of cytochrome c oxidase induces cytochrome c release in anti-Fas-triggered apoptotic cells (Hajek et al., 2001). The release from the mitochondrial intermembrane space into the cytosol involves the activation of the apoptotic protease cascade together with Apaf-1 and procaspase-9 (Li et al., 1997). Interestingly, cytochrome c release from mitochondria is inhibited in the liver of HCV protein-expressing transgenic mice, in which Fas-mediated apoptotic cell death was suppressed (Machida et al., 2001). Nevertheless, the underlying mechanism of cytochrome c release is not well known yet. Further studies on down-regulation of cytochrome c oxidase subunit VIII in relation to respiratory activity of cytochrome c oxidase and cytochrome c release in our experimental setting are necessary.

We characterized the effect of HCV core protein on the cytokine-mediated apoptotic pathway in Hep191 cells. The cytokines Fas ligand and TNF-α are the critical components necessary for triggering receptor-mediated cell death in vivo. We show here that the core protein confers resistance against apoptotic DNA fragmentation induced by either anti-Fas antibody or TNF-α (Figs. 5 and 6). Our data confirmed that the pleiotropic effects of PNA do not cause this phenomenon, since we found no influence on the Fas-mediated apoptosis in control HepRXR cells even with a high concentration of PNA (30 μM). To avoid clonal diversity during the selection of permanent transfectants, we also analyzed HepG2 cells transiently transfected with both core-expressing pIND191 and pVgRXR. We observed similar results of antiapoptotic response (data not shown).

Caspase-3 is recognized as an important effector molecule in the apoptotic pathway, and its activity arises following the cleavage of inactive proenzyme (procaspase-3) after Fas or TNFR is stimulated. We show here that expression of core protein inhibits caspase-3 activity induced by anti-Fas. It was shown by quantifying the enzyme activity in cell extracts and monitoring the level of inactive 32-kDa procaspase-3. Previously some reports indicated that HCV proteins, particularly core protein, are involved in augmentation of caspase activation induced by anti-Fas or TNF-α (Ruggieri et al., 1997; Hahn et al., 2000), whereas others indicated its suppression (Marusawa et al., 1999; Otsuka et al., 2002; Machida et al., 2001). Since different experimental systems were used in these studies, the observed inconsistency may be due to the possible involvement of cellular

factor(s) in certain cells. Alternatively, the expression level of the core protein may be different. A previous report from our laboratory demonstrated that the core protein sensitizes Fas-mediated apoptosis possibly accompanied by up-regulation of caspase-3 (Ruggieri et al., 1997). We used Hep39 cells in that study. Hep39 cells are also originated from HepG2 cells and constitutively expresses the HCV core protein. Subsequently, we compared the steady-state level of the core protein in Hep39 and in Hep191 cells established in this study. The level in Hep39 cells is 100- to 500-fold higher than in Hep191 cells treated with 10 μM PNA determined by FEIA (data not shown).

It is generally accepted that caspase-3 is the primary inactivator of ICAD and is mainly involved in proteolytic pathways that induce apoptotic internucleosomal DNA fragmentation (Wolf et al., 1999). The results in the present study suggest that up-regulation of ICAD and inhibition of caspase-3 activity induced by the core protein collaboratively play roles in blocking DNase activity of CAD and consequently in inhibiting DNA fragmentation in human liver cells, which express the viral protein at moderate levels comparable to that found in patients with chronic liver diseases.

In conclusion, we established a tightly regulated inducible expression system of HCV core protein in human liver cells, where expression of the viral protein is promptly induced to physiological levels. Transcriptional profiling, coupled with studies to confirm gene expression, in this system allowed us to identify several interesting genes that may be involved in cell growth or apoptosis. Evidence that the core protein modulates ICAD expression and the pathway of caspase-3 activation should contribute to the understanding of the molecular mechanisms of HCV persistence and chronic liver diseases. Functional studies in the future to evaluate the significance of potential core-regulated genes implicating not only in apoptosis but in other cellular processes may also provide new directions for the detailed investigation of HCV pathogenesis.

Materials and methods

Construction of inducible expression vector for HCV core protein

HCV cDNA (nt 342 to 914), coding for the entire core protein (aa 1 to 191), was excised from the *Bgl*II site of the pCAGC191 (Suzuki et al., 2001) and inserted, using standard cloning procedures (Sambrook et al., 1989), into the *Bam*HI site of the inducible expression vector pIND/Hygro (Invitrogen, Carlsbad, CA) to yield plasmid pIND191. This vector contains five ecdysone/glucocorticoid response elements which allow binding of the modified VgEcR for activation of gene transcription and inducible expression of HCV core protein gene under control of a Drosophila minimal heat shock promoter. Hygromycin resistance gene in the vector allows selection of stable transfectant in mammalian cells.

Establishment of an inducible cell line

Stock cultures of human hepatoblastoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin and kept at 37°C in a 5% CO_2 incubator. To generate a stable cell line allowing tightly regulated expression of HCV core protein, HepG2 cells were cotransfected with both plasmid pIND191 and pVgRXR (Invitrogen). The latter vector that contains the gene conferring Zeocin resistance also encodes modified ecdysone receptor (VgEcR) and the retinoid X receptor. This vector thus provides an ecdysone functional heterodimeric receptor for activation of gene expression upon binding to an ecdysone analog, such as PNA (Invitrogen) (No et al., 1996).

Monolayers of HepG2 cells were transfected with the plasmids in the presence of Trans-IT LT1 (Mirus Corp. WI) according to the manufacturer's instructions. After transfection, transformants were selected in DMEM containing 500 μg/ml of Hygromycin (Roche Diagnostics, Basel, Switzerland) and 400 μg/ml of Zeocin (Invitrogen) for 3 to 5 weeks. Individual colonies were isolated and amplified, followed by screening inducible expression of HCV core protein. After 24 h of incubating the amplified clones in the medium with PNA at 10 μM, expression of the core protein was determined by a sensitive FEIA (Kashiwakuma et al., 1996) (see below). Stable cell lines, which allow tightly regulated expression of the core protein, were designated as Hep191.

To obtain a control cell line not expressing HCV core protein, HepG2 cells were transfected with the plasmid pVgRXR. After transfection, transformants were selected in DMEM medium containing 400 μg/ml of Zeocin. The isolated resistant colonies were designated as HepRXR.

Quantitation of HCV core protein

Hep 191 cells were induced to express HCV core protein using PNA at various concentrations ranging from 0.3 to 30 μM for indicated periods. Expressed core protein was quantitated by FEIA method as reported previously (Kashiwakuma et al., 1996). Briefly, cells were homogenized in a solution containing 0.3% Triton X-100, 1.5% (3-cholamidopropyl dimethylammonio) propanesulfonic acid, and 15% sodium dodecyl sulfate (SDS). After incubating at 56°C for 30 min, the samples were centrifuged at 1000 rpm for 5 min, and the supernatants were subjected to FEIA. Total protein concentration of the lysates was determined using the Pierce Micro BCA protein Assay Reagent Kit according to the manufacturer's instructions (Pierce, Rockford, IL).

Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed in SDS sample buffer. Cell lysates were separated by 15 or 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to polyvinylidene difluoride membrane (Immobilion; Millipore Ltd., Bredford, MA). After blocking in nonfat milk solution (Block Ace, Yukijirushi Co., Sapporo, Japan), the membranes were probed with the monoclonal antibody against HCV core protein (B2, ANOGEN, Missisauga, Canada), as a primary antibody diluted 1:200 and incubated for 1 h at 37°C. After being washed, the membranes were incubated with horseradish peroxidase conjugated sheep anti-mouse or anti-rabbit immunoglobulins as secondary antibodies. Antigen-antibody complexes were visualized by enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol.

Immunofluorescence microscopy

For indirect immunofluorescence staining of HCV core protein, Hep191 cells were grown in a tissue chamber slide in the presence of 30 μM PNA for 48 h and fixed with 4% paraformaldehyde, followed by permeabilizing with 0.2% Triton X-100. After blocking in nonfat milk solution (Block Ace), cells were incubated for 1 h with the anticore monoclonal antibody. Following washing, cells were incubated for 1 h with Biotin-conjugated antibody to mouse immunoglobulin (Sigma-Aldrich, St. Louis, MO). To increase sensitivity and specificity of the antigen detection, ExtrAvidin conjugated with FITC (Sigma-Aldrich) was then added and incubated for 1 h, followed by examining with a fluorescent microscopy.

Cell growth property

To test cell growth of Hep191, 6×10^4 cells per well were seeded into six-well tissue culture plates. Cells were cultured in DMEM with FBS 10 or 1% and the medium with and without 10 μM PNA was changed daily. Cells from triplicate wells were harvested and viable cells were counted every other day for 10 days by trypan blue dye exclusion.

RT-PCR

Total RNAs were extracted from Hep191 and HepRXR cells using SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturers' protocols. Three micrograms of total RNAs was reverse transcribed by Superscript II (Invitrogen) using oligo(dT) primer. Amplification procedure using specific PCR primers was as follows: after 3 min at 96°C, the reaction of 96°C for 30 s, 55°C for 30 s, 72°C for 1 min was repeated for 20–30 cycles, and finally 10 min at 72°C for one cycle. A minimum amount of PCR cycle was carried out to stay within the linear amplification process for each gene. PCR products were electrophoresed on 2.0% agarose/ethidium bromide gels.

Microarray analysis

Preparation procedure of cDNA microarray was described previously (Yoshikawa et al., 2000; Otsuka et al., 2001). Total RNAs from Hep191 in the presence or absence of PNA at 10 μM for 36 h were isolated with ISOGEN Reagent (Nippon Gene, Tokyo, Japan). Poly(A)$^+$ RNAs were obtained with Oligotex-dT 30 mRNA purification kit (TaKaRa Bio. Inc., Kyoto, Japan), according to the manufacturer's instructions. Fluorescent nucleotide Cy3-dUTP or Cy5-dUTP was incorporated during reverse transcription of the poly(A)$^+$ RNA. The probes were purified and concentrated by passing through Centricon-30 microconcetrators (Millipore). The different fluorescence-labeled probes were mixed and applied onto a microarray slide. Hybridization was carried out at 65°C overnight under a humidified condition. The array was washed, centrifuged, and then scanned with a fluorescence laser confocal slide scanner (Scan Array 4000, GSI Lumonics, Tokyo, Japan). Images were analyzed and fluorescence intensities were measured by Quant Array (GSI Lumonics). Genes showing changes in expression by twofold or more in the average of two experiments, where labeling with the fluorescent dyes were switched during the synthesis, were considered as differentially expressed genes.

Anti-Fas and TNF-α-induced apoptotic response

Hep191 and HepRXR were cultured in the presence of 10 μM PNA for 36 h, followed by treating with anti-Fas antibody (CH-11; MBL, Nagoya, Japan) or recombinant human TNF-α (Sigma-Aldrich). Anti-Fas antibody and TNF-α were used at final concentrations of 100 and 10 ng/ml, respectively. The minimum concentration of cycloheximide (CHX) required for anti-Fas-induced apoptosis was previously determined as a final concentration of 500 ng/ml (Marusawa et al., 1999). After 14 or 48 h from the start of anti-Fas- or TNF-α treatment, respectively, apoptotic cell death was measured by Cell Death Detection ELISAPLUS (Roche Diagnostics) according to the manufacturer's instructions. This assay is based on the specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of apoptosis-induced cells. Anti-Fas-induced apoptotic response in Hep191 was also evaluated using the TUNEL procedure using In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics). Cells incubated in chamber slides were fixed with 4% paraformaldehyde in PBS, followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate. DNA strand breaks generated during apoptosis were fluorescein-labeled and

detected by fluorescence microscopy. Caspase-3 activity in the anti-Fas-treated Hep191 was evaluated by the CaspACETM Assay System (Promega), according to the manufacturer's instructions. Cytosolic proteins were incubated with the colorimetric substrate Ac-DEVD-pNA in the presence or absence of the caspase inhibitor Z-VAD-FMK. Released pNA, *p*-nitroaniline, from the substrate upon cleavage by caspase-3 activity was monitored by a photometer at 405 nm. Total protein concentration of the cell lysates was determined using the Pierce Micro BCA protein Assay Reagent Kit (Pierce).

Luciferase assay

Human embryonic kidney 293T cells in 24-mm-diameter dishes were transfected with 0.5 μg of HCV-core expressing plasmid pCAGC191 or control pCAGGS, 0.2 μg of a reporter plasmid containing ICAD promoter either pIPT-2, -3, or -4 (Kawane et al., 1999), and 0.01 μg of pRL-TK (Promega). After 24 h, cells were harvested, and luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega) as described previously (Aoki et al., 1998). The measured firefly luciferase activities were normalized for transfection efficiency using the values of Renilla luciferase activity determined from the same cell extracts.

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References

Aoki, Y., Aizaki, H., Shimoike, T., Tani, H., Ishii, K., Saito, I., Matsuura, Y., Miyamura, T., 1998. A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. Virology 250, 140–150.

Ashkenazi, A., Dixit, V.M., 1998. Death receptors: signaling and modulation. Science 281, 1305–1308.

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

Dubrez, L., Savoy, I., Hamman, A., Solary, E., 1996. Pivotal role of a DEVD- sensitive step in etoposide-induced and Fas-mediated apoptotic pathways. EMBO J. 15, 5504–5512.

Dumoulin, F.L., von dem Bussche, A., Sohne, J., Sauerbruch, T., Spengler, U., 1999. Hepatitis C virus core protein does not inhibit apoptosis in human hepatoma cells. Eur. J. Clin. Invest. 29, 940–946.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S., 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.

Fujita, T., Ishido, S., Muramatsu, S., Itoh, M., Hotta, H., 1996. Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus. Biochem. Biophys. Res. Commun. 229, 825–831.

Gale Jr., M., Kwieciszewski, B., Dossett, M., Nakao, H., Katze, M.G., 1999. Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resis. PG. J. Virol. 73, 6506–6516.

Hahn, C.S., Cho, Y.G., Kang, B.S., Lester, I.M., Hahn, Y.S., 2000. The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. Virology 276, 127–137.

Hajek, P., Villani, G., Attardi, G., 2001. Rate-limiting step preceding cytochrome c release in cells primed for Fas-mediated apoptosis revealed by analysis of cellular mosaicism of respiratory changes. J. Biol. Chem. 276, 606–615.

Hayashi, N., Mita, E., 1997. Fas system and apoptosis in viral hepatitis. J. Gastroenterol. Hepatol. 12, S223–226.

Honda, M., Kaneko, S., Shimazaki, T., Matsushita, E., Kobayashi, K., Ping, L.H., Zhang, H.C., Lemon, S.M., 2000. Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells. Hepatology 31, 1351–1359.

Hong, N.A., Flannery, M., Hsieh, S.N., Cado, D., Pedersen, R., Winoto, A., 2000. Mice lacking Dad1, the defender against apoptotic death-1, express abnormal N-linked glycoproteins and undergo increased embryonic apotosis. Dev. Biol. 220, 76–84.

Kashiwakuma, T., Hasegawa, A., Kajita, T., Takata, A., Mori, H., Ohta, Y., Tanaka, E., Kiyosawa, K., Tanaka, T., Tanaka, S., Hattori, N., Kohara, M., 1996. Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J. Immunol. Methods 190, 79–89.

Kawane, K., Fukuyama, H., Adachi, M., Sakahira, H., Copeland, N.G., Gilbert, D.J., Jenkin, N.A., Nagata, S., 1999. Structure and promoter analysis of murine CAD and ICAD genes. Cell Death Differ. 6, 745–752.

Koike, K., Tsutsumi, T., Fujie, H., Shintani, Y., Kyoji, M., 2002. Molecular mechanism of viral hepatocarcinogenesis. Oncology 62 (Suppl. 1), 29–37.

Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., et al., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244, 362–364.

Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., Joulin, V., Kahn, A., 1996. Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. Nat. Med. 2, 80–86.

Lai, M.M.C., Ware, C.F., 1999. Hepatitis C virus core protein: possible roles in viral pathogenesis, in: Hagedorn, C.H., Rice, C.M. (Eds.), The Hepatitis C Viruses, Springer, Berlin, pp. 117–134.

Lau, J.Y., Xie, X., Lai, M.M., Wu, P.C., 1998. Apoptosis and viral hepatitis. Semin. Liver Dis. 18, 169–176.

Li, K., Prow, T., Lemon, S.M., Beard, M.R., 2002. Cellular response to conditional expression of hepatitis C virus core protein in Huh7 cultured human hepatoma cells. Hepatology 35, 1237–1246.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf- 1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.

Liu, X., Zou, H., Slaughter, C., Wang, X., 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89, 175–184.

Machida, K., Tsukiyama-Kohara, K., Seike, E., Tone, S., Shibasaki, F., Shimizu, M., Takahashi, H., Hayashi, Y., Funata, N., Taya, C., Yonekawa, H., Kohara, M., 2001. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. J. Biol. Chem. 276, 12140–12146.

Marusawa, H., Hijikata, M., Chiba, T., Shimotohno, K., 1999. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J. Virol. 73, 4713–4720.

Moradpour, D., Englert, C., Wakita, T., Wands, J.R., 1996. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. Virology 222, 51–63.

Nagata, S., 2000. Apoptotic DNA fragmentation. Exp. Cell Res. 256, 12–18.

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S., Nishimoto, T., 1993. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell. Biol. 13, 6367–6374.

No, D., Yao, T.P., Evans, R.M., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA 93, 3346–3351.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., Nagata, S., 1993. Lethal effect of the anti- Fas antibody in mice. Nature 364, 806–809.

Otsuka, M., Kato, N., Taniguchi, H., Yoshida, H., Goto, T., Shiratori, Y., Omata, M., 2002. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. Virology 296, 84–93.

Otsuka, M., Kato, M., Yoshikawa, T., Chen, H., Brown, E.J., Masuho, Y., Omata, M., Seki, N., 2001. Differential expression of the L-plastin gene in human colorectal cancer progression and metastasis. Biochem. Biophys. Res. Commun. 289, 876–881.

Patel, T., Roberts, L.R., Jones, B.A., Gores, G.J., 1998. Dysregulation of apoptosis as a mechanism of liver disease: an overview. Semin. Liver Dis. 18, 105–114.

Ray, R.B., Meyer, K., Ray, R., 1996. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 226, 176–182.

Ray, R.B., Meyer, K., Steele, R., Shrivastava, A., Aggarwal, B.B., Ray, R., 1998. Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. J. Biol. Chem. 273, 2256–2259.

Ruggieri, A., Harada, T., Matsuura, Y., Miyamura, T., 1997. Sensitization to Fas- mediated apoptosis by hepatitis C virus core protein. Virology 229, 68–76.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.-L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87, 6547–6549.

Sakahira, H., Enari, M., Nagata, S., 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391, 96–99.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Suzuki, R., Suzuki, T., Ishii, K., Matsuura, Y., Miyamura, T., 1999. Processing and functions of Hepatitis C virus proteins. Intervirology 42, 145–152.

Suzuki, R., Tamura, K., Li, J., Ishii, K., Matsuura, Y., Miyamura, T., Suzuki, T., 2001. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 280, 301–309.

Tai, D.I., Tsai, S.L., Chen, Y.M., Chuang, Y.L., Peng, C.Y., Sheen, I.S., Yeh, C.T., Chang, K.S., Huang, S.N., Kuo, G.C., Liaw, Y.F., 2000. Activation of nuclear factor kappaB in hepatitis C virus infection: Implications for pathogenesis and hepatocarcinogenesis. Hepatology 31, 656–664.

Tanaka, K., Kondoh, N., Shuda, M., Matsubara, O., Imazeki, N., Ryo, A., Wakatsuki, T., Hada, A., Goseki, N., Igari, T., Hatsuse, K., Aihara, T., Horiuchi, S., Yamamoto, N., Yamamoto, M., 2001. Enhanced expression of mRNAs of antisecretory factor-1, gp96, DAD1 and CDC34 in human hepatocellular carcinomas. Biochem. Biophys. Acta 1536, 1–12.

Thomson, M., Liang, T.J., 2000. Molecular biology of hepatitis C virus, in: Liang, T.J., Hoofnagle, J.H. (Eds.), Hepatitis C, Academic Press, San Diegopp, pp. 1–24.

Tsutsumi, T., Suzuki, T., Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Matsuura, Y., Kimura, S., Koike, K., Miyamura, T., 2003. Alteration of intrahepatic cytokine expression and AP-1 activation in transgenic mice expressing hepatitis C virus core protein. Virology 00, 000–000.

Wolf, B.B., Schuler, M., Echeverri, F., Green, D.R., 1999. Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor- 45/inhibitor of caspase-activated DNase inactivation. J. Biol. Chem. 274, 30651–30656.

Yoshikawa, T., Nagasugi, Y., Azuma, T., Kato, M., Sugano, S., Hashimoto, K., Masuho, Y., Muramatsu, M., Seki, N., 2000. Isolation of novel mouse genes differentially expressed in brain using cDNA microarray. Biochem. Biophys. Res. Commun. 275, 532–537.

Yoshimi, M., Sekiguchi, T., Hara, N., Nishimoto, T., 2000. Inhibition of N-linked glycosylation causes apoptosis in hamster BHK21 cells. Biochem. Biophys. Res. Commun. 276, 965–969.

Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., Lai, M.M., 1998. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. J. Virol. 72, 3691–3697.

Zhu, N., Ware, C.F., Lai, M.M., 2001. Hepatitis C virus core protein enhances FADD-mediated apoptosis and suppresses TRADD signaling of tumor necrosis factor receptor. Virology 283, 178–187.

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Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor

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Abstract

Lack of efficient culture systems for hepatitis C virus (HCV) has been a major obstacle in HCV research. Human liver cells grown in a three-dimensional radial-flow bioreactor were successfully infected following inoculation with plasma from an HCV carrier. Subsequent detection of increased HCV RNA suggested viral replication. Furthermore, transfection of HCV RNA transcribed from full-length cDNA also resulted in the production and release of HCV virions into supernatant. Infectivity was shown by successful secondary passage to a new culture. Introduction of mutations in RNA helicase and polymerase regions of HCV cDNA abolished virus replication, indicating that reverse genetics of this system is possible. The ability to replicate and detect the extracellular release of HCV might provide clues with regard to the persistent nature of HCV infection. It will also accelerate research into the pathogenicity of HCV, as well as the development of prophylactic agents and new therapy.

Keywords: HCV; Three-dimensional radial-flow bioreactor; Replication; Full-length cDNA; Infectious clone; Particles; Reverse genetics; Infection; In vitro culture model; Artificial liver

Introduction

More than 100 million people are infected with hepatitis C virus (HCV) worldwide, and many are prone to developing chronic hepatitis with subsequent cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). Measures to eliminate HCV from carriers before progressive and degenerative liver diseases develop are needed. However, studies aimed at elucidating the mechanism behind persistent HCV infection have been hampered by lack of an efficient in vitro culture system capable of supporting viral replication.

Many attempts have been made to culture HCV in vitro. However, to date, only limited HCV replication was suggested by reverse transcription-polymerase chain reaction (RT-PCR) techniques, and HCV-specific proteins were not generally detected (Ito et al., 2001; Kato and Shimotohno, 1999; Thomson and Liang, 2000). Infectious cDNA clones have been successfully developed for positive-sense RNA viruses (Boyer and Haenni, 1994). Full-length HCV cDNA clones have also been successfully used to infect chimpanzees in in vivo transfection experiments (Kolykhalov et al., 1997, 2000; Yanagi et al., 1997). Furthermore, replication of genome-length dicistronic HCV RNA was shown in a

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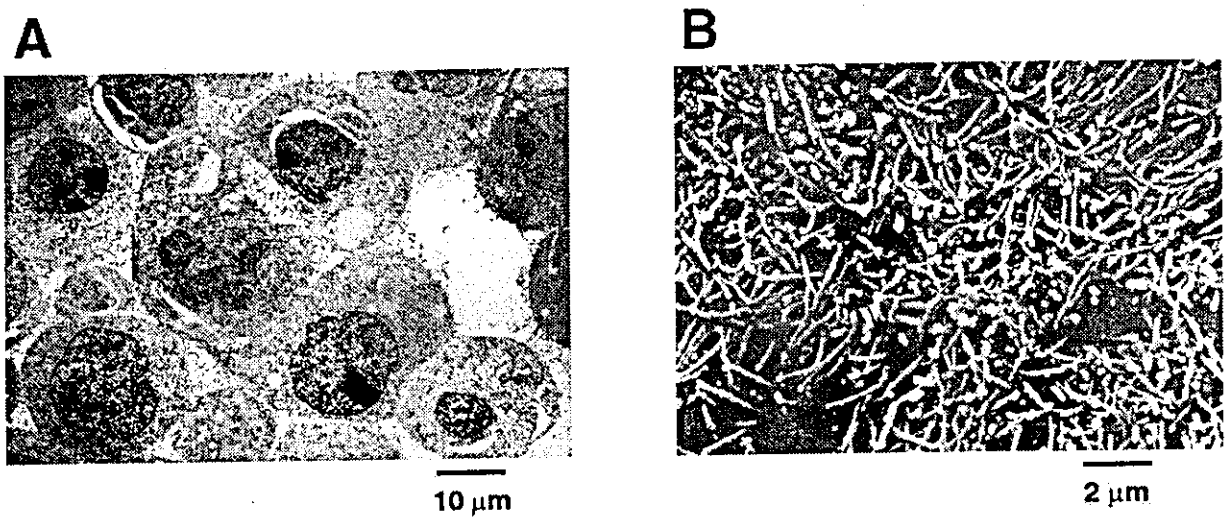

Fig. 1. FLC4 cells in the RFB. (A) Transmission electron micrograph image of FLC4 cells cultured in the RFB. Scale bar shows 10 μm. (B) Scanning electron micrographs showing FLC4 cells attached with the RFB matrix. Scale bar shows (B).

human liver cell line when HCV RNA was dicistronically transfected together with selective markers (Ikeda et al., 2002; Pietschmann et al., 2002). Although replication of the entire HCV genome was also observed, any infectious HCV virions could not be detected (Pietschmann et al., 2002). Thus, viral RNA replication and synthesis of fully processed viral proteins may not be sufficient to produce infectious virus. One or more host factor(s) provided only by permissive cells may be required for the assembly of infectious virions. Alternatively, monolayer cultures of mammalian cells, typically human liver-derived cell lines, may not permit the assembly and release of HCV from cells.

Nagamori et al. established a three-dimensional (3D) radial-flow bioreactor (RFB) system, in which human liver cells retained their differentiated hepatocyte functions and morphological appearance for an extended period of time (Kawada et al., 1998). This system was originally designed to develop artificial liver tissue (Kawada et al., 1998; Matsuura et al., 1998). However, in this study, we utilized the system to examine cells transfected with full-length HCV RNA or inoculated with infectious serum. Although the titer was not particularly high, propagation of HCV in vitro was clearly shown. Furthermore, infectious virus was released into the supernatant in the absence of obvious cell lysis.

Results

FLC4 cell culture in RFB system

We found that cells from a human hepatocellular carcinoma-derived cell line, FLC4 (Functional Liver Cell 4) (Aoki et al., 1998), produced albumin at a rate of 18 $\mu g/10^5$ cells/day, when cultured in the RFB system. In conventional monolayer cultures, FLC4 cells produced albumin at a rate of 2.5 $\mu g/10^5$ cells/day.

α-Fetoprotein production by FLC4 cells also differed among the two culture systems (7 $\mu g/10^5$ cells/day were produced by cells in the RFB system versus an undetectable amount by cells in the monolayer culture). Production of albumin and α-fetoprotein by FLC4 cells in the RFB culture continued for more than 100 days without cell passage, during which time the temperature was reduced from 37 to 32°C, causing a gradual increase in oxygen consumption.

Transmission electron microscopy (TEM) has shown maintenance of tight junctions among RFB-cultured FLC4 cells and normal intercellular spaces (Fig.1A). Scanning electron microscopy (SEM) has shown that, unlike FLC4 cells in monolayer cultures, FLC4 cells cultured in the RFB system retain structurally intact microvilli on their surface (Fig. 1B). RFB culture is therefore thought to provide an environment in which the natural architecture and function of cells are maintained. We tested the replication of HCV under these conditions as a model of HCV replication in vivo.

Infection experiments

First, we inoculated FLC4 cells with infectious human plasma in RFB culture. The infectious plasma (no. 6) was derived from a healthy HCV carrier and its infectivity was eventually proven after posttransfusion hepatitis C was observed in a patient that had received the carrier's blood (Takeuchi et al., 1990; Aizaki et al., 1998). Afterward, the infectivity of the carrier's plasma was tested by inoculation of chimpanzees, and its titer was determined to be $10^{5.5}$ chimpanzee infectious doses per milliliter (Sugitani and Shikata, 1998).

Table 1
HCV infection of FLC4 cells cultured in the RFB following inoculation with the plasma

Days	HCV RNA[a] ($\log_{10}$ copies/ml)	ALT (IU/l)
−2	−	7
0[b]	5	2
1	+	NT[c]
2	−	NT
3	−	2
4	−	NT
5	−	2
7	−	NT
9	−	2
11	−	NT
12	−	2
14	−	NT
16	+	2
19	4–5	2
23	4	2
26	+	2

[a] HCV RNA in the culture fluids was determined by qualitative and quantitative RT-PCR. Log of copy numbers identified by the quantitative assay are shown. +, positive by the qualitative assay but below the detection limit (10^3 copies/ml) by the quantitative one. −, negative by both of assays.
[b] On the day 0, plasma no. 6 was inoculated.
[c] NT, not tested.

After cells (1×10^9) were inoculated with 1 ml of the plasma in RFB culture by infusion, HCV RNA was detected by PCR in the culture fluid for 2 days. This was thought to be due to residual virus following the infusion. RNA was not detected in the culture fluid for the next 2 weeks (less than 50 copies/ml). HCV RNA was then detected on day 16 postinfection (p.i.) and reached $10^4 - 10^5$ copies/ml by days 19 and 23 p.i. (Table 1). After this, HCV RNA was detected in the culture fluid for more than 80 days by the qualitative assay (data not shown). Subsequent experimentation using the same cells and inoculum reproduced this profile of HCV RNA detection in the culture fluid (data not shown). In this experiment, the cells were harvested on day 14, and further culture was discontinued. Intracellular HCV core protein levels were as high as 75 pg/mg of total cellular protein in the cells. Although the precise number of cells supporting virus replication could not be determined, this level of core protein expression is almost equivalent to that observed in the liver tissue of chronic hepatitis C patients (Koike et al., 2002).

The hypervariable regions (HVR) of HCV RNA detected on day 23 p.i. were compared to those of plasma no. 6 (Table 2). Plasma no. 6 (donor plasma) was found to contain a mixture of clones with different HVR sequences, but more than half of these contained A1 sequences (Sugitani and Shikata, 1998). After transmission, these mixed clones converged until one clone was primarily detected (clone A1) in infected chimpanzees (Chimp.) (Sugitani and Shikata, 1998), and in the human hepatitis patient, who received no. 6 plasma for transfusion (Recip.) (Aizaki et al., 1998). Interestingly, of the 20 clones tested in this RFB culture study, 19 had A1 clone sequences. These data suggest that the HCV RNA detected was not that of the infused virus but, rather, it appears that the RFB culture selectively supported the replication of certain clones. Clones having A1 sequences may have a replication advantage in RFB culture.

Table 2
Comparison of amino acid sequences of HVR among HCV clones derived from plasma no. 6

Species	Sequence of HVR[a]	Number of clones			
		Donor[b]	Chimp.	Recip.	RFB
	384 410				
A1	H Y R V T R G V Q G H V T S T L T S L F R P G A S Q K	11	20	2	19
A2	S -[c]	1	0	0	0[c]
A3	- L - - -	1	0	0	0
A4	- F - -	0	0	0	1
B1	N - H -	1	0	0	0
B2	N - H - - - A - - - - G A F G - - - - - Q - - - - - -	3	0	0	0
B3	N - H - - - A - - - - G A F - - - - - - T S - - - - -	0	0	1	0
B4	D - H - - - A - - - - G A F - - - - - - T S - - - - -	3	0	0	0
B5	D - H - - - A - - - - G A F Q - - - - - T S - - - - -	0	0	1	0
B6	D - H - - - A - - - - G A F Q - - - - - T S - - - - R	0	0	1	0
B7	D - H - - - A - - - - G A F H - - - - - T S - - - - -	0	0	2	0
B8	D - H - - - A - - C - G A F H - - - - - T S - - - - -	0	0	1	0
C1	N - H - - - - - - - R - A - K - - - F - T - - P - - -	0	0	1	0
D1	- - H - - - - - - - - - - - - - - - F K - S - - - - -	0	0	1	0
		20	20	10	20

[a] Sequences of HVR in HCV clones recovered from the culture fluid of the RFB (RFB), the plasmas of a donor (Donor), a chimpanzee (Chimp.), and a recipient (Recip.) are shown.
[b] The sequences and diversity in Donor, Chimp., and Recip. are from Sugitani and Shikata (1998), and Aizaki et al. (1998).
[c] Dashes represent amino acid residues identical to the clone A-1.

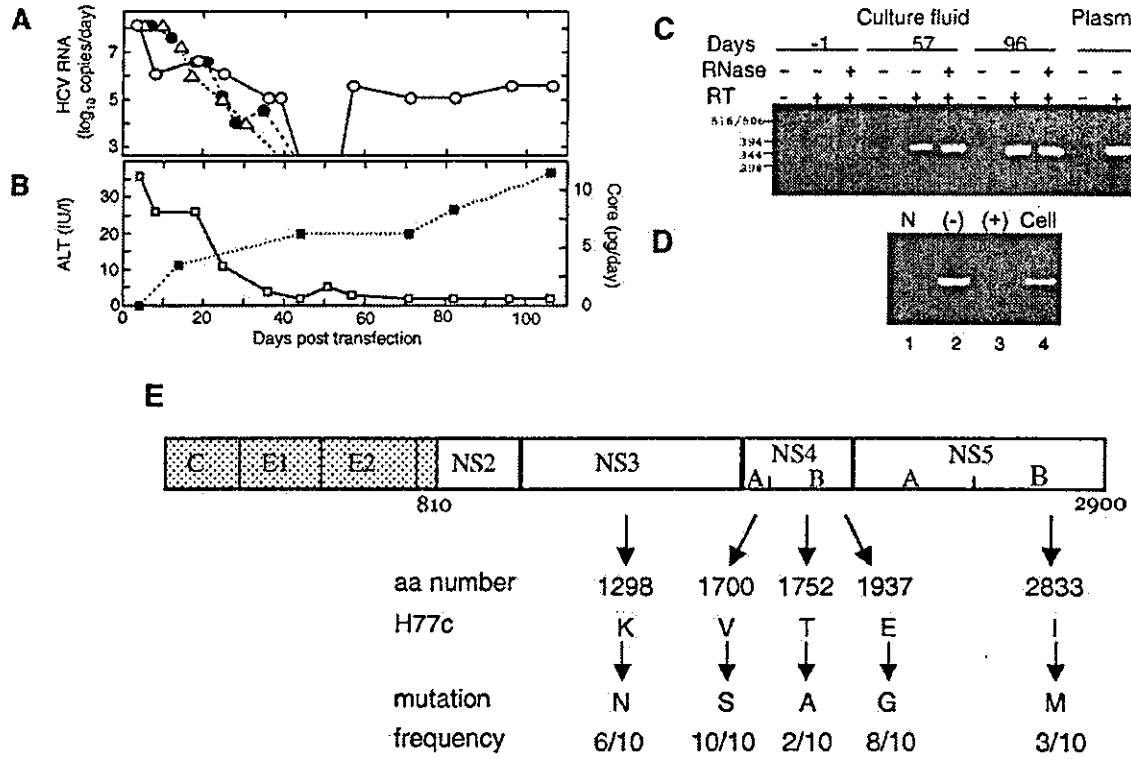

Fig. 2. HCV propagation in FLC4 cells in RFB culture following transfection with the RNA derived from the infectious cDNA clone. (A) HCV RNA titers in the culture fluid after transfection of the infectious HCV RNA H77c (○), helicase inactive mutant RNA H77cHel- (△), and RdRp inactive mutant RNA H77cRdRp- (●). On the day 0, the full-length HCV RNA H77c was transfected. HCV RNAs at days 44, 47, 51, and 54 were positive, but their RNA titers were $<10^3$ copies/ml. (B) ALT levels (□) and the amount of HCV core protein (■) in the culture fluid after transfection of H77c, respectively. (C) Culture fluids of the H77c transfectant were treated with (+) or without (−) RNase A. Samples were then amplified by RT-PCR with (+) or without (−) enzyme in the RT step. The culture fluids collected on day −1, 57, and 96 after inoculation, patient plasma, and the cell-culture medium added with 6×10^4 molecules of HCV RNA transcript were analyzed. (D) Negative-strand-specific RT-PCR, Lane 1, cellular RNA without added synthetic RNA. Lanes 2 and 3, with added synthetic negative (−) and positive (+)-strand RNA, respectively. Lane 4, cellular RNA from the cells harvested on the day 110 p.t. (E) HCV clones recovered from the culture fluids of the RFB. (Top) Schematic presentation of the complete HCV genome. The ORF with the structural proteins (shaded box) located in the amino-terminal portion of polyprotein, and the remainder encodes the nonstructural protein (NS2 to NS5B). (Middle) The positions and frequencies. Overlapping RT-PCR products were sequenced and the mutations observed in nonstructural protein regions are illustrated, along with the frequency with each arose. The amino acid number is indicated above the H77c sequence.

Transfection experiments

The above results suggest that RFB culture supports the replication and release of HCV in culture fluid. To confirm this, we transfected cells in RFB culture with HCV RNA. RNA derived from an infectious full-length HCV cDNA clone (H77c) (Yanagi et al., 1997) was used. This clone was derived from HCV genotype 1a and was proven infectious by direct inoculation into chimpanzee livers.

Cells were transfected with HCV RNA and cultured using the RFB system. HCV RNA was assayed by RT-PCR using samples of culture fluid. As in the infection experiments described above, the amount of viral RNA in the culture fluid declined immediately after transfection. At days 44, 47, 51, and 54 posttransfection (p.t.), HCV RNA was positive by the qualitative assay but below the detection limit (10^3 copies/day) by the quantitative one (Aizaki et al., 1998). However, the sample collected on day 57 p.t. indicated HCV RNA levels as $10^5 - 10^6$ copies/day. This level remained here for 100 days of culture (Fig. 2A, open circles and solid line). In addition, HCV core protein gradually increased in the culture fluid until a maximum level of approximately 13.2 pg/day was reached on day 106 p.t. (Fig. 2B, filled squares and dotted line). The relative amounts of HCV core protein (13.2 pg/day) and HCV RNA ($10^5 - 10^6$ copies/day) detected on day 106 p.t. are consistent with previously reported data on native HCV virions (Kashiwakuma et al., 1996). Since ALT levels did not increase within the culture fluid (Fig. 2B, open squares and solid line), direct hepatocyte injury did not appear to result from HCV replication.

To determine whether the HCV RNA detected was packaged within virions, the culture fluid collected on days 57 and 96 p.t. was treated with RNase and examined by quantitative RT-PCR. As shown in Fig. 2C, the HCV nucleic acid was first confirmed to be RNA. However, the RNA was RNase resistant, suggesting that it is contained within virions. The RNase resistance might also indicate HCV RNA of