incubated at 32 °C. In the case of HCV, cells cultured at 32 °C generally become more susceptible to viral infection and replication, and the infectivity and stability of HCV produced by cells cultured at 32 °C is greater [25]. Twenty-four, 48, and 72 h after the infection, the cells were then prepared for extension of the DNA samples.

2.4. *Interaction of bLF and hLF with cells or virus*

To clarify the target sites of LF for anti-HBV activity, we examined the LF interaction with either the cells or HBV or both. We first examined the interaction of bLF (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and hLF (Sigma, Chemical Co., St. Louis, MO) with the cells, and PH5CH8 cells (1×10^5) were plated in a 24-well microtiter plate and cultured for 2 days before viral inoculation. bLF was added to the cells (in 500 μl of medium) at a final concentration of 0.1 or 1.0 mg/ml and incubated for 60 min at 37 °C. Free bLF was removed by washing twice with 1 ml of F-12 (Invitrogen Corp., Carlsbad, CA). After the addition of 500 μl of medium containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After the incubation, free HBV was removed by washing twice with 1 ml of PBS (Fig. 1A). Next, to examine the interaction of bLF with HBV, 10 μl of HBV serum and bLF (final concentration of 0.1 or 1.0 mg/ml) was preincubated in 500 μl of medium for 60 min at 4 °C, the mixture of HBV and bLF was then added to PH5CH8 cells cultured as described above, and incubated for 210 min at 32 °C. After the incubation, free HBV was removed by washing twice with 1 ml of PBS (Fig. 1B), and the cells were then prepared for extension of the DNA samples.

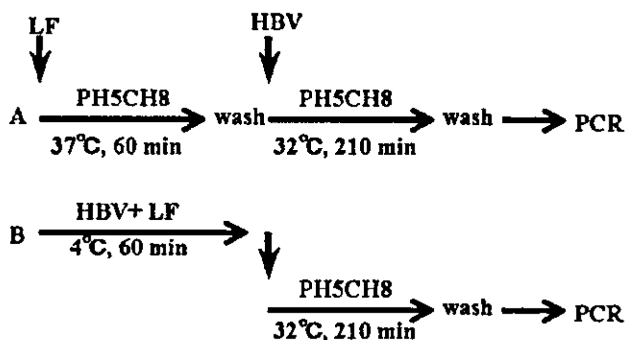

Fig. 1. The method of bLF interaction with cells or viruses. (A) PH5CH8 cells and bLF (final concentration of 0.1 or 1.0 mg/ml) were preincubated for 60 min at 37 °C, free bLF was removed and HBV serum (10 μl) was added and incubated for 210 min at 32 °C. Free HBV was removed by washing twice with 1 ml of F-12. (B) HBV serum (10 μl) and bLF (final concentration of 0.1 or 1.0 mg/ml) were preincubated for 60 min at 4 °C, the mixture was added to PH5CH8 cells and incubated for 210 min at 32 °C.

2.5. *Anti-HBV activities of bTF, casein and LA*

To clarify the specificity of the anti-HBV activity of bLF, we further examined the anti-HBV effect of another iron transporter, bovine transferrin (bTF), casein, the main protein included in human milk, and lactoalbumin (LA, Wako Pure Chemical Industries, Ltd., Osaka, Japan). PH5CH8 cells (1×10^5) were cultured for 2 days at 37 °C before viral inoculation. bTF, casein and LA were added to the cells (in 500 μl of medium) at a final concentration of 2.0 mg/ml and severally incubated for 60 min at 37 °C. In order to exclude the possibility of a lack of any result being due to a low dose, the concentration of each reagent was increased from 1.0 to 2.0 mg/ml. After the incubation, free bLF, casein and LA were removed by washing twice with 1 ml of F-12. After the addition of 500 μl of medium containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After the incubation, free HBV was removed by washing twice with 1 ml PBS. The cells were then prepared for extension of the DNA samples.

3. Results

3.1. *Anti-HBV effect of bLF: interaction of bLF with the cells*

Cloned PH5CH8 cells were found to be a human hepatocyte line susceptible to HCV infection [19]. In this study, we found that PH5CH8 cells could support HBV infection with reduction

Fig. 2. Adhesion of HBV to cells. PH5CH8 cells (1×10^5) were plated and cultured at 37 °C for 2 days before virus inoculation. After addition of 500 μl of F-12 containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After free HBV had been removed and the cells were washed twice with PBS, cells were cultured at 32 °C. Seventy two h after virus inoculation, the HBs region of HBV DNA derived from the cells was amplified by nested PCR. HBV DNA was detectable in 11 of 12 wells (lines B–F, and H–M). Rluc was detectable in all lines. A, Marker; N, positive control; O, negative control.

of the temperature from 37 to 32 °C during the adsorption period.

As shown in Fig. 2, 24, 48, and 72 h after infection of the cells with HBV, HBV DNA was detectable from HBV-adsorbed PH5CH8 cells in 11 of 12 wells. Using this HBV infection system, we examined whether bLF protects cultured hepatocytes against HBV infection. To clarify the target sites of bLF for anti-HBV activity, we examined the interaction of bLF with either the cells or HBV, or even both. As shown in Fig. 3, HBV DNA was undetectable from HBV-adsorbed PH5CH8 cells pretreated with bLF (1.0 mg/ml), as well as being undetectable in one of the duplicate DNA samples, when the concentration of bLF was 0.1 mg/ml. The PCR system was reliable because Rluc was detectable in all PCR products. These results suggest that bLF interacts with the cells and prevents adsorption of HBV into the cells.

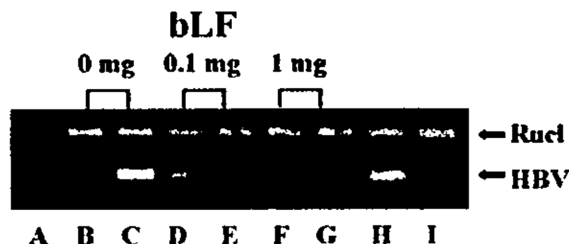

Fig. 3. Anti-HBV effect of bLF: interaction of bLF with cells. PH5CH8 cells (1×10^5) were plated and cultured at 37 °C for 2 days before virus inoculation, in duplicate. bLF was added to the cells at a final concentration of 0.1 or 1.0 mg/ml and incubated for 60 min at 37 °C, and the cells were then washed twice with 1 ml of F-12. After addition of 500 μl of F-12 containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After free HBV had been removed and the cells were washed twice with PBS, the HBs region of HBV DNA adhering to the cells was amplified by nested PCR. HBV DNA was detectable without bLF pretreatment (lines B and C), but was undetectable in one of the duplicate DNA samples in which PH5CH8 had been pretreated with 0.1 mg/ml of bLF (lines D and E). HBV DNA was completely undetectable when the concentration of bLF was 1 mg/ml (lines F and G). Rluc was detectable in all lines. A, Marker; H, positive control; I, negative control.

3.2. *Anti-HBV effect of bLF: interaction of bLF with virus*

On the contrary, Fig. 4 demonstrates HBV DNA from cells is detectable following bLF pretreatment of HBV positive serum. The PCR system was reliable because Rluc was detectable in all PCR products. This result suggests that pretreating HBV serum with bLF does not effectively inhibit HBV infection. From these results, it can be concluded that interaction of bLF with the cells is essential for the prevention of HBV infection.

3.3. *Anti-HBV effect of hLF: interaction of hLF with cells*

With regards to the anti-HBV effect of hLF, HBV DNA was not detectable in PH5CH8 cells

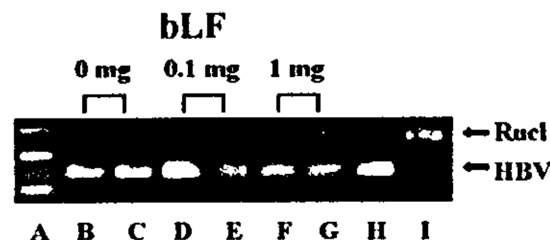

Fig. 4. Anti-HBV effect of bLF: interaction of bLF with virus. F-12 (500 μl) containing 10 μl of HBV serum and bLF (0.1 or 1.0 mg/ml) were preincubated for 60 min at 4 °C. This mixture was then added to the PH5CH8 cell culture system and incubated for 210 min at 32 °C. After incubation, cells were removed and HBV DNA from infected cells was amplified by nested PCR. HBV DNA was detectable with or without bLF pretreatment (lines B–G). The concentrations of bLF were 0 (lines B and C), 0.1 mg/ml (lines D and E), and 1 mg/ml (lines F and G). Rluc was detectable in all lines. A, Marker; H, positive control; I, negative control.

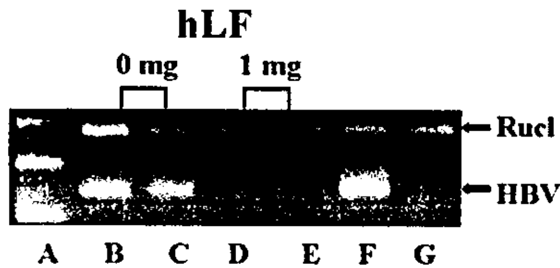

Fig. 5. Anti-HBV effect of hLF: interaction of hLF with cells. hLF was added to the cells at a final concentration of 1.0 mg/ml and incubated for 60 min at 37 °C, and then the cells were washed twice with 1 ml of F-12. After addition of F-12 (500 μl) containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After free HBV had been removed and the cells washed, the HBs region of HBV DNA was amplified by nested PCR HBV DNA was detectable without hLF pretreatment (lines B and C), but was undetectable when PH5CH8 was pretreated with 1 mg/ml of hLF (lines D and E). Rluc was detectable in all lines. A, Marker; F, positive control; G, negative control.

pretreated with hLF (1.0 mg/ml), and was undetectable in one of the duplicate DNA samples, when hLF was used at the 0.1 mg/ml concentration (Fig. 5). The PCR system was reliable because Rluc was detectable in all PCR products. This result indicates that the anti-HBV activity of LF is not species-specific dependent.

3.4. Anti-HBV effect of LF: specificity of anti-HBV activity of LF

To clarify the specificity of the anti-HBV activity of bLF, we examined the anti-HBV effects of another iron transporter, bTF, and another milk protein, casein, and LA. As shown in Fig. 6, bTF, casein and LA showed no activity against HBV infection, while bLF did have anti-HBV activity. The PCR system was reliable because Rluc was detectable in all PCR products. The result suggests that the anti-HBV activity associated specifically with LF is not a general property of the iron transporter family proteins and milk proteins.

4. Discussion

LF has antiviral effects against HIV, HCMV [17], and HSV-1 [9], based on *in vitro* studies, and the interaction of LF with cells has been shown to be important for its antiviral activities. LF is also known to have antiviral activities against HCV infection [18], and the interaction of bLF with HCV is responsible for the inhibitory effect. In this study, we found for the first time that LF is one of the candidates for an anti-HBV agent *in vitro*, and the mechanism of the inhibitory effect of LF on HBV infection in our study is same as that of human HIV-1, HCMV and HSV-1, but different from that of HCV.

Several other potential mechanisms by which LF may inhibit the growth of several microorganisms have been suggested, including structural changes in the microbial cell wall, complete loss of membrane potential, indirect effects on enzyme activation, increased generation of metabolic by-products of aerobic metabolism, iron deprivation and combinations of these factors [26–30]. Although the antiviral activity of LF has been well described, the mechanism of its antiviral action is poorly characterized. In the case of

Fig. 6. Anti-HBV effect of LF: specificity of anti-HBV activity of LF. bLF (final concentration of 1.0 mg/ml), bovine transferrin (bTF), casein (CA) or lactoalbumine (LA) (final concentration of 2.0 mg/ml) was added to the cells and incubated for 60 min at 37 °C, and the cells were then washed twice with 1 ml of F-12. After the addition of 500 μl of F-12 containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After free HBV has been removed and the cells washed, the HBs region of HBV DNA adhering to the cells was amplified by nested PCR. HBV DNA was detectable without bLF pretreatment (lines B and C), but was undetectable when pretreated with 1 mg/ml of bLF (lines D and E). However, it was detectable when pretreated with 2 mg/ml of bTF (lines F and G), CA (lines H and I) and LA (lines J and K). Rluc was detectable in all lines. A, Marker; L, positive control; M, negative control.

HCV infection, LF could interact directly with the envelope protein of HCV, in such a way that LF may neutralize the HCV virion [31]. In the case of HBV infection, 1-h pretreatment of cells with bLF was effective, while pretreatment of HBV with bLF was not, therefore, LF interacts with cell surface protein and blocks the viral adhesion to the cells.

LF has been known to have several receptors, such as a single-chain receptor of 105 kDa on activated T cells [32] and three 37-kDa subunits on intestinal brush border membranes [13]. There are some reports indicating that LF exerts two distinct types of binding inhibition on hepatocytes. The first mechanism involves the relationship between bLF and lipoprotein. LF has been observed to be an antagonist of apolipoprotein E [33]. In this case, LF attached directly to the cell surface receptor, low density lipoprotein (LDL) receptor related protein (LRP), and blocks the interaction of apolipoprotein E with its receptor. Alternatively, LF can function as an inhibitor of cholesterol accumulation in macrophages [34]. In this case, LF binds directly to oxidized-LDL and blocks its binding to scavenger receptors on macrophages. The second mechanism involves LF possibly interacting with heparin and various glycosaminoglycans [35,36]. McAbee et al. reported that LF binds to the asialoglycoprotein receptor in rat liver [37]. Asialoglycoprotein has been proposed as a possible receptor for HBV [38], therefore, the latter mechanism may be a plausible explanation of the inhibitory effect of LF in the case of HBV.

Although the overall structure of LF is very similar to that of TF, LF and TF are distinguished by a few features that may be important functionally. The affinity of LF for iron is 250-fold greater than that of TF [39]. Furthermore, in comparison to TF, the most unique region of LF is the N-terminal region, which forms a loop structure, designated lactoferricin [40]. This loop structure contains a basic amino acid cluster and is presumed to be responsible for both bactericidal activity [40] and binding activities to LRP, as the antagonist of apolipoprotein E [33], and to oxidized-LDL [34]. More lipopolysaccharide is released by lactoferricin than by whole bovine LF, and whereas bovine LF is at most bacteriostatic, lactoferricin has consistent bactericidal activity against gram-negative bacteria [15]. Lactoferricin H (residues 1–47) and lactoferricin B (residues 17–41) are released by pepsinolysis of human and bovine LF, respectively, and may have a more potent antibacterial activity than the native proteins [40]. Furthermore, the N-terminal 33 residues of human LF reportedly represent the minimal sequence that mediates binding of the protein to glycosaminoglycans [41]. This sequence contains a cationic head (residues 1–6) and tail (residues 28–33), which combine to form the glycosaminoglycan-binding site. In addition, it is possible that the glycosaminoglycan receptor is the HBV receptor on the hepatocyte plasma membrane [42]. Therefore, LF may weakly adhere to a molecule on the cell surface, such as heparin, as well as to the asialoglycoprotein receptor and glycosaminoglycan receptor. This would allow LF to mechanically block the normal process of viral adhesion.

Further investigation is required before any conclusions regarding the LF region responsible for anti-HBV activity, and the effectiveness of LF in patients with HBV infection can be drawn. Recently, we reported that LF inhibits hepatitis C viremia in hepatitis due to chronic hepatitis C [43]. Therefore, there is the possibility that LF could be effective for treating HBV infection in humans.

References

[1] Vogel CL, Anthony PP, Sadikali F, Barker LF, Peterson MR. Hepatitis-associated antigen and antibody in hepatocellular carcinoma: results of a continuing study. J Natl Cancer Inst 1972;48:1583–8.

[2] Obata H, Hayashi N, Motoike Y, et al. A prospective study on the development of hepatocellular carcinoma from liver cirrhosis with persistent hepatitis B virus infection. Int J Cancer 1980;25:741–7.

[3] Szmuness W. Hepatocellular carcinoma and the hepatitis B virus: evidence for a causal association. Prog Med Virol 1978;24:40–69.

[4] Summers J, O'Connell A, Millman I. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc Natl Acad Sci USA 1975;72:4597–601.

[5] Robinson WS. The genome of hepatitis B virus. Annu Rev Microbiol 1977;31:357–.

[6] Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. Coli*. Nature 1979;281:646–50.

[7] Colacino JM, Staschke KA. The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. Prog Drug Res 1998;50:259–322.

[8] Ono-Nita SK, Kato N, Shiratori Y, et al. YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: A study by in vitro full-length viral DNA transfection. Hepatology 1999;29:939–45.

[9] Hasegawa K, Motsuchi W, Tanaka S, Dosako S. Inhibition with lactoferrin of in vitro infection with human herpes virus. Jpn Med Sci Biol 1995;47:73–85.

[10] Bullen JJ, Rogers HJ, Griffiths E. Role of iron in bacterial infection. Curr Top Microbiol Immunol 1987;80:1–35.

[11] Cohen MS, Britigan BE, French M, Bean K. Preliminary observations on lactoferrin secretion in human vaginal mucus: variation during the menstrual cycle, evidence of hormonal regulation, and implication for infection with *Neisseria gonorrhoeae*. Am J Obstet Gynecol 1987;157:1122–5.

[12] Masson PL, Heremans LF, Dive CH. An iron-binding protein common to many external secretions. Clin Chim Acta 1966;14:735–9.

[13] Lonnerdal B, Iyer S. Lactoferrin: molecular structure and biological function. Annu Rev Nutr 1995;15:93–110.

[14] Spik G, Strecker G, Fournet B, et al. Primary structure of the glycans from human lactoferrin. Eur J Biochem 1982;121:413–9.

[15] Yamauchi K, Tomita M, Giehl TJ, Elison RT, III. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect Immun 1993;61:719–28.

[16] Kirkpatrick CH, Green I, Rich RR, Schade AL. Inhibition of growth of *Candida albicans* by iron-unsaturated lactoferrin: relation to host defense mechanisms in chronic mucocutaneous Candidasis. J Infect Dis 1971;124:539–44.

[17] Harmsen MC, Swart PJ, deBethune MP, et al. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. J Infect Dis 1995;172:380–8.

[18] Ikeda M, Sugiyama K, Tanaka T, et al. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. Biochem Biophys Res Commun 1998;245:549–53.

[19] Noguchi M, Hirohashi S. Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In vitro cell. Dev Biol Anim 1996;32:135–7.

[20] Kato N, Ikeda M, Mizutani T, et al. Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. Jpn J Cancer Res 1996;87:787–92.

[21] Ikeda M, Kato N, Mizutani T, Sugiyama K, Tanaka K, Shimotohno K. Analysis of the cell tropism of HCV by using in vivo HCV-infected human lymphocytes and hepatocytes. J Hepatol 1997;27:445–54.

[22] Kato N, Sekiya H, Ootsuyama Y, et al. Humoral immune response to hypervariable region 1 of the putatice envelope glycoprotein (gp70) of hepatitis C virus. J Vilol 1993;67:3923–30.

[23] Mizutani T, Ikeda M, Saito S, Sugiyama K, Shimotohno K, Kato N. Single-step reverse transcription-polymerase chain reaction for the detection of hepatitis C virus RNA. Microbiol Immunol 1998;42:544–53.

[24] Nozaki A, Naganuma A, Nakamura T, Tanaka K, Sekihara H, Kato N. A reliable internally controlled RT-Nested PCR Method for the detection of hepatitis C virus RNA. Acta Med (Okayama) 2000;54:253–7.

[25] Mizutani T, Kato N, Ikeda M, Sugiyama K, Shimotono K. Long-term human T cell culture system supporting hepatitis C virus replication. Biochem Biophys Res Commun 1996;277:822–6.

[26] Zhang GH, Mann DM, Tsai CM. Neutralization of endotoxin in vitro and in vivo by a human lactoferrin-derived peptide. Infect Immun 1999;67:1353–8.

[27] Appelmelk BJ, An YQ, Geerts M, et al. Lactoferrin is a lipid A-binding protein. Infect Immun 1994;62:2628–32.

[28] Cole AM, Dewan P, Ganz T. Innate antimicrobial activity of nasal secretions. Infect Immun 1999;67:3267–75.

[29] Schibli DJ, Hwang PM, Vogel HJ. The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. FEBS Lett 1999;446:213–7.

[30] Kuipers ME, deVries HG, Eikelboom MC, Meijer DK, Swart PJ. Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical Candida isoletes. Antimicrob Agents Chemother 1999;43:2635–41.

[31] Yi M, Kaneko S, Yu DY, Murakami S. Hepatitis C virus envelope proteins bind lactoferrin. J Virol 1997;71:5997–6002.

[32] Mazurier J, Legrand D, Hu WL, Montreuil J, Spik G. Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography. Eur J Biochem 1989;179:481–7.

[33] van Dijk MC, Ziere GJ, van Berkel TJ. Characterization of the chylomicron-remnant-recognition sites on parenchymal and Kupffer cells of rat liver. Selective inhibition of parenchymal cell recognition by lactoferrin. Eur J Biochem 1992;205:775–84.

[34] Kajikawa M, Ohta T, Takase M, Kawase K, Shimamura S, Matsuda I. Lactoferrin inhibits cholesterol accumulation in macrophages mediated by acetylated or oxidized low-density lipoproteins. Biochim Biophys Acta 1994;1213:82–90.

[35] Damiens E, El Tazidi I, Mazurier J, et al. Role of heparan sulphate proteoglycans in the regulation of human lactoferrin binding and activity in the MDA-MB-231 breast cancer cell line. Eur J Cell Biol 1998;77:344–51.

[36] Shimazaki K, Tazume T, Uji K, et al. Properties of a heparin-binding peptide derived from bovine lactoferrin. J Dairy Sci 1998;81:2841–9.
[37] McAbee DD, Bennatt DJ, Ling YY. Identification and analysis of a CA (2+) –dependent lactoferrin receptor in rat river. Lactoferrin binds to the asialoglycoprotein receptor in a galactose-independent manner. Adv Exp Med Biol 1998;443:113–21.
[38] Treichel U, Meyer zumBuschenfelde KH, Dienes HP, Gerken G. Receptor-mediated entry of hepatitis B virus particles into liver cells. Arch Virol 1997;142:493–8.
[39] Brock J. Lactoferrin: a multifunctional immunoregulatory protein. Immunol Today 1995;16:417–9.
[40] Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K, Tomita M. Identification of the bactericidial domain of lactoferrin. Biochim Biophys Acta 1992;1121:130–6.
[41] Mann DM, Romm E, Migliorini M. Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. J Biol Chem 1994;269:23661–7.
[42] Atkins GJ, Qiao M, Coombe DR, Gowans EJ, Ashman LK. Hepatitis B virus binding to leucocyte plasma membranes utilizes a different region of the preS1 domain to the hepatocyte receptor binding site and does dot require receptors for opsonins. Immunol Cell Biol 1997;75:259–66.
[43] Tanaka K, Ikeda M, Nozaki A, Kato N, Tsuda H, Saito S, Sekihara H. Lactoferrin inhibits hepatitis C virus viremia in patients with chronic hepatitis C: a pilot study. Jpn J Cancer Res 1999;90:367–71.

J Gastroenterol 2002; 37[Suppl XIII]:50–54

Journal of Gastroenterology

© Springer-Verlag 2002

Hepatitis C virus and its roles in cell proliferation

Kunitada Shimotohno, Koichi Watashi, Katsuya Tsuchihara, Katsuhiko Fukuda, Hiroyuki Marusawa, and Makoto Hijikata

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan

Hepatitis C virus (HCV) causes chronic hepatitis and is linked to the development of hepatocellular carcinoma (HCC). The role of HCV infection in the development of HCC remains to be clarified. We analyzed the effect of HCV core protein on modulation of cell proliferation. HCV core protein was shown to have at least two functions: activation of the Ras/Raf signaling pathway and anti-apototic function.

Key words: apoptosis, hepatocellular carcinoma, hepatitis C virus, infalmmation, MAP kinase, NFκB

Introduction

Chronic infection by hepatitis C virus (HCV) leads to slowly progressive liver disease that over a period of up to 30 years may result in cirrhosis, chronic liver failure, and perhaps hepatocellular carcinoma (HCC). Patients with more active and severe liver disease seem to be at higher risk of developing cancer, whereas healthy carriers of HCV have little risk of HCC. It has been suggested that the severity of inflammation is an important factor determining the prognosis of the development of HCC.[1] Acceleration of DNA synthesis of hepatocytes, which is frequently correlated with severe inflammation, seems to be one factor influencing the development of HCC.

Inflammation is related to clearance of HCV-infected cells by activated cytotoxic T cells (CTLs), and therefore it is assumed that the level of inflammation is correlated with CTL activity. Although it is likely that immunological surveillance by CTLs is the main source of inflammation, additional factors may also be involved. One such factor may be a direct cytopathic effect of HCV infection itself on cells. Anther possibility is that HCV replication itself may affect inflammation caused by CTLs. In this case, HCV proteins may be involved in modifying inflammation quantitatively or qualitatively. In this report, the possible role of HCV proteins, in particular core protein, in the modulation of cell proliferation is described.

HCV core protein is encoded at the most 5′-terminal region of the HCV open reading frame (ORF) and is produced by cleavage between amino acids 191 and 192 of the precursor polyprotein by host signal peptidase.[2] In addition to the encapsidation of viral RNA, recent in vitro studies suggest that HCV core protein has other biological properties, such as modulation of transcription and regulation of apoptosis.[3–11] Although the mechanisms of these regulations are still unclear, it is possible that these properties of HCV core protein influence host cell growth, survival, and carcinogenesis. It was also shown that HCV core protein has transforming potential in rat embryo fibroblasts with or without active *ras* gene products,[4,12] and transgenic mice expressing HCV core gene developed hepatocellular carcinoma.[13] However, the precise role of HCV core protein in these transformation processes remains to be elucidated. Therefore, we wish to clarify how HCV core protein contributes to the development of transformation.

Materials and methods

Plasmids

pCMV-Core, which encodes a polypeptide spanning from amino acids 1 to 191 of the HCV precursor polyprotein under the control of the cytomegalovirus immediate-early promoter, has been described previously.[14] To construct a human Elk1-expressing plasmid, pCMV-Elk1, a cDNA of human Elk1, was obtained by

Reprint requests to: K. Shimotohno

reverse transcription–polymerase chain reaction (RT-PCR) from total RNA of HeLa cells and subcloned in frame into a mammalian expression vector with the cytomegalovirus immediate-early promoter (pCMV-KS). The following plasmids were purchased from Stratagene (La Jolla, CA, USA): pFA-Elk, which contains the C-terminal transactivation domain of Elk1 fused to the Gal4 DNA binding domain; pFR-Luc, which contains the luciferase coding region under the control of five tandem repeats of the Gal4 DNA-binding sequence; pSRE-Luc, which contains the luciferase reporter gene driven by a basic promoter element joined to five tandem repeats of SRE binding elements; and pFC-MEK1, which encodes constitutively active MEK1. pSR-HA-MAPK, which encodes HA-tagged *Xenopus* MAPK; pSR-HA-SAPK, which encodes HA-tagged *Xenopus* SAPK; and pSR-HA-p38, which encodes HA-tagged *Xenopus* p38 MAPK, were kind gifts from Dr. Eisuke Nishida (Kyoto University), and pSR-RasN17, which encodes a dominant negative form of Ras, was kindly provided by Dr. Shin Yonehara (Kyoto University). Other plasmids used in this work are described elsewhere.

Evaluation of cell death

Apoptotic cell death was evaluated by determining cell viability, caspase activation, and the detection of DNA fragmentation. The cells transfected with plasmids were treated with anti-Fas antibody (CH-11, MBL, Nagoya, Japan) or recombinant human tumor necrosis factor α (TNF-α) (Sigma, St. Louis). Anti-Fas and TNF-α were used at final concentrations of 100ng/ml and 10ng/ml, respectively. The number of dead cells was counted as those stained with trypan blue dye within four microscopic fields 14h or 48h from the start of anti-Fas or TNF-α treatment, respectively. Apoptotic cell death was also measured by Cell Detection enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostic, Tokyo, Japan) according to the manufacturer's protocol.

Results and discussion

Cooperative transformation of BALB/3T3 A31-I-1 cells by HCV core protein and v-H-ras gene products

One of the subclones of BALB/3T3 cells, A31-I-1, is known to be transformed by expression of H-Ras plus c-myc.[15,16] BALB/3T3 A31-I-1 cells were transfected with pLXSH-core191, which is an expression plasmid of the HCV core gene encoding 191 amino acids, with or without v-H-ras expression plasmid, pLXSN-ras. Several focus-forming colonies were obtained at 21 days post-transfection. The number of focuses was equivalent to that observed in experiments performed with *myc* and *ras* genes, whereas transfection with pLXSH-core191 alone did not produce any foci. Cloned Bcr cells, expressing HCV core protein and H-Ras, showed altered morphology and a disoriented pattern in culture dishes and lost contact inhibition of growth.[17] This result suggested that HCV core protein transformed BALB/3T3 A31-I-1 cells cooperatively with v-H-ras gene product.

HCV core protein contributes to the acquisition of growth advantages of cells

First, we prepared other BALB/3T3-derived cell lines, Bc and Br, stably transfected with LXSH, LXSH-core191, and LXSN-ras, respectively. Then, we compared the growth rates or Bmr cells expressing c-Myc and H-Ras with those of Bcr, Bc, and Br cells. Cell proliferation was measured by MTT assay at 24-h intervals. The Bcr cells proliferated in serum-free medium as efficiently as Bmr cells. Bc and Br cells did not show any growth under the same conditions. We supposed that HCV core protein contributed to the acquisition of growth advantages of BALB/3T3 cells cooperatively with active Ras.[17]

Because Ras is activated by the stimuli of growth factors, we examined whether HCV core protein enhances cell growth in growth factor–supplemented medium. Bc and the original cells were cultured in serum-free media supplemented with 0, 0.1, and 1.0μg/ml of epidermal growth factor (EGF) for 72h, and the proliferation of cells in each condition was measured by MTT assay. Bc and the original cells did not proliferate in serum-free media, and the original cells did not respond to EGF stimuli. Bc cells were sensitive to EBF stimuli and grew effectively. It is suggested that HCV core does not stimulate cell proliferation without growth stimuli, but cooperates with growth factors or their intracellular downstream signals and enhances cell growth, at least in BALB/3T3 A31-I-1 cells. Bc cells did not show any growth advantages in a medium supplemented with 10% fetal bovine serum (FBS).

HCV core protein activated the MEK-ERK pathway

In response to mitogenic signals, Ras activates Raf in a GTP-dependent manner. To determine how HCV core protein enhances activation of Elk1 in response to mitogenic signals, cells were transfected with pFR-Luc, pFA-Elk1, and pSR-RasN17, which encodes dominant negative Ras, together with pCMV-Core. Twenty-four hours after transfection, luciferase assays showed that the expression of dominant negative Ras completely inhibited the activation of Elk1 in either the presence or absence of HCV core protein. Expression of both dominant negative Ras and constitutively active MEK1

activated Elk1. Under these conditions, HCV core protein increased the activation of Elk1.

To investigate the phosphorylation status of exogenous expressed HA-tagged MAPK, Western blot analysis using phospho-specific antibody against MAPK was performed. Immunostaining of Western blots confirmed that exogenous HA-tagged MAPK was equally phosphorylated in HCV core-expressing cells and control cells. There was no significant difference in the amount of phospho-ERK 1/2 in cells expressing HCV core protein and in control cells. These data indicate that HCV core protein does not influence the phosphorylation and activation of ERK.

There was no significant difference in the amount of either phospho-Elk1 or total expressed Elk1 between HCV core-expressing cells and control cells.

These data indicate that HCV core protein does not affect Elk1 phosphorylation, suggesting that HCV core protein does not influence the traditional phosphorylation cascade itself, which begins with Ras activation and evokes Elk1 activation. HCV core protein may enhance Elk1 activation by a phosphorylation-independent, still uncharacterized mechanism.

HCV core protein protected the cells from Fas- and TNF-α–mediated apoptotic cell death, possibly through activation of NFκB

HepG2 cells transfected with pCMV-Core for production of the core showed increased viabilty after treatment with anti-Fas. The anti-apoptotic effect of the core was observed in different cell types treated with anti-Fas. MCF-7 cells producing the core also showed a high cell survival ratio in comparison with negative control cells (68.0 ± 4.1% vs. 49.5 ± 2.3%, respectively) against TNF-α–induced apoptosis. Thus, we concluded that the core was responsible for suppressing the apoptotic response mediated by either Fas or TNF-α in different cell lines.

To determine whether core production affects NF-κB activity in the cells, a reporter plasmid assay, in which NF-κB–dependent reporter gene expression was monitored in the presence or absence of HCV proteins, was performed using HepG2 cells cotransfected with pNFκB-Luc and pCMV-Core. Two- and three-fold augmentation of relative luciferase activities was observed as compared with the negative control cells. Furthermore, the treatment of core-producing HepG2 cells with either TNF-α or anti-Fas for 2h resulted in 22- and 17-fold increases in luciferase activity, respectively, whereas the same treatments augmented the reporter activity by about 5-fold in the negative control cells.

C-terminally truncated core abrogated NF-κB activation and had no effect on suppression of apoptotic cell death.

The primary structure of the final core product after secondary processing in cells is still unclear. There have been controversial reports from the results of deletion analysis suggesting that its C-terminal end is located at around 151 or 173 amino acids.[18,19] It was also shown that the subcellular localization of the core was shifted from the cytoplasm to the nucleus by these deletion.[20,21] HepG2 cells transfected with the expression plasmids for the two truncated core proteins, pCMV-ΔCore173 and pCMV-ΔCore151, were analyzed for the ability to activate NFκB. The maximal effect on NF-κB activation was observed in HepG2 cells transfected with pCMV-Core, whereas the cells producing the C-terminal truncated core of 151 amino acids showed no activation of NF-κB. In the HepG2 cells transfected with pCMV-ΔCore173, intermediate effects on NF-κB activation were observed.

This result suggests that the C-terminus of core protein is directly involved in NF-κB activation. Alternatively, the C-terminus may be important for retention of core protein in the perinuclear membrane fraction, where it may function fully to activated NF-κB, and thus the terminus of the core, per se, may not be essential for activation. This possibility was tested by making a fusion protein of core protein in which the C-terminal region was substituted by a peptide with a hydrophobic amino acid cluster, or by a peptide with a nuclear export signal, so that the fused protein would reside on the perinuclear membrane or be diffusely localised in the cytoplasm, respectively. The core-fused protein residing on the membrane activated NF-κB at the highest level, suggesting that it is necesary for core protein to be associated with the perinuclear membrane to activate NF-κB.

The above results suggested that at least one of the anti-apoptotic effects of the core protein is achieved through the activation of NF-κB.

Possible roles of HCV proteins in liver dysfunction

HCV is the major causative agent of chronic liver disease and the development of HCC. However, the mechanisms responsible for liver cell injury remain to be clarified. Persistent infection with these viruses is particularly important for disease progression. Cytoplasmic T lymphocytes play crucial roles in the clearance of viral infection. Recently, it has been demonstrated that perforin- and Fas-based mechanisms account for all T-cell–mediated cytotoxicity. In HCV infection, Fas expression in hepatocytes is up-regulated in accordance with the severity of liver inflammation. When HCV-specific T cells migrate into hepatocytes and recognize the viral antigen via the T-cell receptor, they become activated and express Fas ligand that can transduce the apoptotic death signal to Fas-bearing

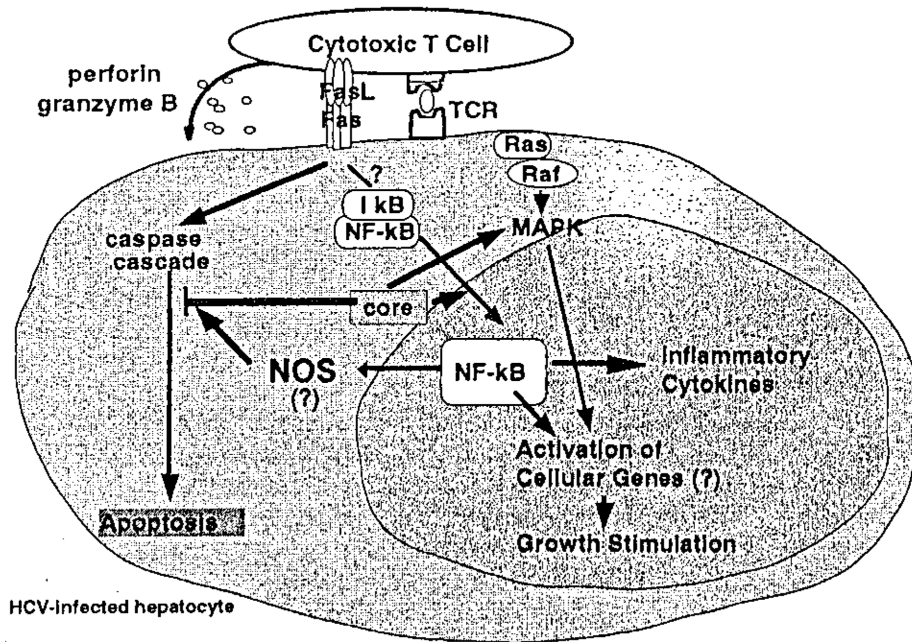

Fig. 1. Hepatitis C virus (HCV)-infected cells may modulate cell proliferation through modification of apoptotic signaling and activation of the Ras/Raf signaling pathway by core protein. *NOS*, Nitric oxide synthase; *TCR*, T-cell receptor; *IκB*, inhibitor factor kappa B; *NF-κB*, nuclear factor kappa B; *MAPK*, mitogen activated protein kinase; *FasL*, Fas ligant

hepatocytes.[22] Thus, the Fas system plays an important role in liver-cell injury by HCV infection. Also, Fas ligand was detected in liver-infiltrating mononuclear cells. These results were consistent with the previous observation that cell killing of hepatocytes by CTLs is mediated by the Fas/Fas ligand signaling pathway (Fig. 1). Up-regulation of Fas and Fas ligand in hepatocytes and mononuclear cells, respectively, suggests enhancement of Fas-mediated apoptosis during CTL surveillance. It is also conceivable that cells in which NF-κB is activated by core protein may suppress Fas-mediated apoptosis, as described here. Such cells may escape from apoptosis and contribute to virus replication and release of virus particles. Persistent infection with HCV may be explained by such a mechanism, in addition to insufficient activation of CTL to clear HCV-infected cells. Because on hepatic infiltration with mononuclear cells hepatocytes may be exposed to many genotoxic agents, such as oxygen radicals, perforin, and granzyme, these hepatocytes may have a high probability of chromosomal mutation. Thus, cells that have escaped from immune surveillance may have a higher chance of developing a malignant phenotype. Core protein also has the potential to activate the mitogen-activated protein kinase (MAPK) cascade, which may have a mitogenic effect. The liver undergoes persistent regeneration following hepatic injury, and growth factors stimulate this liver regeneration in hepatitis. It is possible that HCV core protein in regenerating hepatocytes enhances growth stimuli, and repeated hepatocyte proliferation may cause disorders of genes in hepatocytes, thus causing HCC. In addition, we showed that reduction of HCV core protein production in some cell lines cancels the growth advantage. These observations will help to elucidate the viral pathogenesis and to develop treatment regimens for HCC following HCV infection.

Acknowledgments. This work was supported by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research, a grant-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health and Welfare, by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, and by a grant-in-aid of Research for the Future from the Japan Society for the Promotion of Science, Japan.

References

1. Tarao K, Rino Y, Ohkawa S, Shimizu A, Tamai S, Miyakawa K, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. Cancer 1999;86:589–95.
2. Clarke B. Molecular virology of hepatitis C virus. J Gen Virol 1977;78:2397–2410.
3. Ray RB, Lagging LM, Meyer K, Steele R, Ray R. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. Virus Res 1955;37:209–20.
4. Chang J, Yang H, Cho YG, Hwang SB, Hahn YS, Sung YC. Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. J Virol 1998;72:3060–5.
5. Shrivastava A, Manna SK, Ray R, Aggarwal BB. Ectopic expression of hepatitis C virus core protein differentially regulates nuclear transcription factors. J Virol 1998;72:9722–8.

6. Ray RB, Steele R, Meyer K, Ray R. Transcriptional repression of p53 promoter by hepatitis C virus core protein. J Biol Chem 1997;272:10983–6.
7. Ray RB, Steele R, Meyer K, Ray R. Hepatitis C virus core protein represses $p21^{WAF1/Cip1/Sid1}$ promoter activity. Gene 1998;208:331–6.
8. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176–82.
9. Ray RB, Meyer K, Steele R, Shrivastava A, Aggarwal BB, Ray R. Inhibition of tumor necrosis factor (TNF-α)-mediated apoptosis by hepatitis C virus core protein. J Biol Chem 1998;273:2256–9.
10. Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 1997;229:68–76.
11. Zhu N, Khoshnan A, Schneider R, Matsumoto M, Dennert, G, Ware C, et al. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. J Virol 1998;72:3691–7.
12. Ray RB, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol 1996;70:4438–43.
13. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nature Med 1998;4:1065–7.
14. Marusawa H, Hijikata M, Chiba T, Shimotohno K. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J Virol 1999;73:4713–20.
15. Kakunaga T, Crow JD. Cell variants showing differential susceptibility to ultraviolet light-induced transformation. Science 1980;209:505–7.
16. Sueoka E, Sueoka N, Okabe S, Komori A, Suganuma M, Kozu T, et al. Tumourigenicity of MTG8, a leukaemia-related gene, in concert with v-Ha- *ras* gene in BALB/3T3 cells. Br J Haematol 1998;10:737–42.
17. Tsuchihara K, Hijikata M, Fukuda K, Kuroki T, Yamamoto N, Shimotohno K. Hepatitis C virus core protein regulates cell growth and signal transduction pathway transmitting growth stimuli. Virology 1999;258:100–7.
18. Lo SY, Masiarz F, Hwang SB, Lai MM, Ou JH. Differential subcellular localization of hepatitis C virus core gene products. Virology 1995;213:455–61.
19. Santolini E, Migliaccio E, La Monica N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J Virol 1994;68:3631–41.
20. Liu Q, Tackney C, Bhat RA, Prince AM, Zhang P. Regulated processing of hepatitis C virus core protein is linked to subcellular localization. J Virol 1997;71:657–62.
21. Suzuki R, Matsuura Y, Suzuki T, Ando A, Chiba J, Harada S, et al. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. J Gen Virol 1995;76:53–61.
22. Hayashi N, Mita E. Fas system and apoptosis in viral hepatitis. J Gastroenterol Hepatol 1997;12:S223–6.

Int. J. Cancer: **99**, 681–688 (2002)
© 2002 Wiley-Liss, Inc.

Publication of the International Union Against Cancer

WNT/β-CATENIN SIGNALING SUPPRESSES APOPTOSIS IN LOW SERUM MEDIUM AND INDUCES MORPHOLOGIC CHANGE IN RODENT FIBROBLASTS

Yoshihide UEDA[1], Makoto HIJIKATA[1]*, Shinji TAKAGI[1], Ritsuko TAKADA[2], Shinji TAKADA[2,3], Tsutomu CHIBA[4] and Kunitada SHIMOTOHNO[1]

[1]*Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan*
[2]*Center for Molecular and Developmental Biology, Graduate School of Science, Kyoto University, Kyoto, Japan*
[3]*Kondoh Differentiation Signaling Project, ERATO, Japan Science and Technology Corporation (JST), Kyoto, Japan*
[4]*Division of Gastroenterology and Hepatology, Department of Medicine, Postgraduate School of Medicine, Kyoto University, Kyoto, Japan*

Wnt/β-catenin signaling plays important roles in tumorigenesis in certain tumors as well as during development. However, the mechanism of tumorigenesis mediated by this signaling remains to be elucidated. We investigated the response of rodent fibroblasts to activation of Wnt/β-catenin signaling by treatment with conditioned medium containing soluble Wnt-3a protein (W3a-CM) and by expression of a constitutive active β-catenin gene harbored by an adenovirus vector. W3a-CM induced transcriptional activation of a β-catenin/T-cell factor (Tcf)-responsive promoter in rodent fibroblasts such as NIH3T3, Rat-1, Swiss3T3 and Balb3T3 cells. In these cells, an increase in saturation density and an inhibition of apoptosis and/or promotion of growth in low-serum medium were induced by treatment with W3a-CM. In Rat-1 cells, morphologic changes were also induced. All these alterations were reversible. Moreover, the inhibition of apoptosis of NIH3T3 cells in low-serum medium and the morphologic changes in Rat-1 cells, but not the increase in saturation density, were also induced by ectopic expression of a constitutive active β-catenin gene. These results suggested that activation of Wnt/β-catenin signaling induces inhibition of apoptosis and morphologic changes in these cells.
© *2002 Wiley-Liss, Inc.*

Key words: *β-catenin; Wnt-3a; apoptosis; rodent fibroblast*

The Wnt gene family encodes secreted proteins that play important roles in cell growth, differentiation, organogenesis and oncogenesis.[1,2] Their signal transduction pathways are thought to be functionally conserved in vertebrates and invertebrates. The Wnt gene family consists of a set of 15 or more related genes.[1]

In vertebrates, the Wnt signaling pathway consists of an intracellular cascade that includes Frizzled, Dvl, glycogen synthase kinase-3β (GSK-3β), β-catenin and T-cell factor (Tcf)/lymphocyte enhancer binding factor (Lef). Adenomatous polyposis coli protein (APC) and Axin negatively regulate this signaling by forming a complex with Dvl, GSK-3β and β-catenin. In the absence of Wnt, GSK-3β is active and represses downstream elements of the Wnt signaling pathway through a decrease in the cytoplasmic β-catenin level. In the presence of Wnt, GSK-3β is inactivated through Dvl, there is a decrease in the phosphorylation of β-catenin and an increase in its stability and β-catenin is translocated to the nucleus. In the nucleus, β-catenin binds to Tcf/Lef, a transcription factor and stimulates gene expression. Thus, Wnt induces the accumulation of cytoplasmic β-catenin and transmits the transcription-regulating signal to the nucleus.[3]

In addition to its role in development, the Wnt signaling pathway has been shown to be involved in a number of cancers,[4–7] including colon cancer, melanoma, hepatocellular carcinoma, hepatoblastoma, ovarian cancer, endometrial cancer, medulloblastoma, pilomatricoma and prostate cancer. Mutations of the β-catenin, APC or Axin gene appear to be a crucial step in the progression of a subset of these cancers.[6,7] In addition, in heterozygous ApcD716 knockout mice and in mice overexpressing N-terminally truncated β-catenin, which is stable and transcriptionally active, multiple intestinal adenomas develop.[8,9] These findings show that activation of Wnt/β-catenin signaling has an important role in tumorigenesis *in vivo*. *In vitro* as well as *in vivo*, mutant β-catenin displays transforming activity in NIH3T3 cells, RK3E cells and Madin-Darby canine kidney (MDCK) epithelial cells.[10–12] Furthermore, the expression of LEF-1 fused to the transactivation domain of either VP16 or the estrogen receptor results in the oncogenic transformation of chicken embryo fibroblasts, suggesting that β-catenin/Tcf targets genes that are critical for tumorigenesis. Recently c-myc, cyclin D1, WISP-1, PPARδ and other genes were identified as transcriptional targets of the β-catenin/Tcf complex.[13–16]

However, it is not clear how the phenotype(s) of cells is altered to the malignant state in response to Wnt/β-catenin signaling. Several studies have provided information about alterations to cell growth and death caused by Wnt/β-catenin signaling,[17–21] but different results were obtained in different experiments. For example, the expression of a molecule contained in the Wnt/β-catenin signaling pathway had various effects on apoptosis. In NIH3T3 cells, apoptosis was induced by overexpression of β-catenin.[18] Induction of apoptosis by activation of β-catenin during development was suggested by the following observations: inactivation of the *Drosophila* homolog of APC induced apoptosis of the *Drosophila* retinal neurons (an effect that was mimicked by overexpression of Armadillo[22]) and mutation of presenilin-1 (which is a major cause of familial early-onset Alzheimer's disease) induced apoptosis of neural cells via stabilization of β-cate-

Abbreviations: Ad-β-cat, adenovirus expressing the myc-tagged mutant β-catenin; Ad-LacZ, adenovirus expressing the b-galactosidase gene; APC, adenomatous polyposis coli protein; +FCS, DMEM with 10% FCS only; GSK-3β, glycogen synthase kinase-3β; IFN-β BP, interferon-β binding protein; L-CM, conditioned medium from L cells; Lef, lymphocyte enhancer binding factor; L+FCS, DMEM with 10% FCS containing L-CM; L-FCS, DMEM containing L-CM; MOI, multiplicity of infection; Tcf, T-cell factor; W+FCS, DMEM with 10% FCS containing W3a-CM; W-FCS, DMEM containing W3a-CM; W3a-CM, conditioned medium containing soluble Wnt-3a protein; wt, wild type.

Grant sponsor: Ministry of Health and Welfare of Japan (Grants-in-Aid for Cancer Research and the Second-Term Comprehensive 10-Year Strategy for Cancer Control); Grant sponsor: Ministry of Education, Science and Culture (Grants-in-Aid for Scientific Research); Grant sponsor: Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan (Program for the Promotion of Fundamental Studies in Health Sciences).

*Correspondence to: Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.
Fax: +81-75-751-3998. E-mail: mhijikat@virus.kyoto-u.ac.jp

Received 27 August 2001; Revised 24 January 2002; Accepted 6 March 2002

DOI 10.1002/ijc.10418

nin.[23] In contrast, another group reported that expression of β-catenin in MDCK cells suppressed suspension-induced apoptosis (anoikis).[20] Furthermore, the finding that the expression of APC or Axin in human colorectal cancer cells and hepatoma cells induced apoptosis, or that PC12 cells expressing Wnt-1 exhibited less apoptosis in the absence of serum, suggested that activated β-catenin has a role in the suppression of apoptosis.[24–26] Thus, these results differed depending on the cells analyzed and on the way in which β-catenin was activated.

Changes in the growth and morphology of cells induced by the exogenous expression of Wnt, β-catenin, Axin or APC were also reported.[17,19–21,25,27] In the studies, in which β-catenin was expressed exogenously, it was unclear whether the level expressed was relevant to the level in cancer cells that have mutations in the β-catenin, APC or Axin gene, or in cells at the developmental stage at which β-catenin is activated by Wnt. It was reported that different quantities of a transcription factor resulted in different effects.[28] Moreover, reports in which exogenous Wnt, APC or Axin was expressed in cells[24–26] did not make it clear whether the alterations in cells actually depended on the activation of β-catenin.

To identify the phenotypical changes induced by Wnt/ β-catenin signaling, we first investigated the growth, death and morphology of rodent fibroblasts, which are known to be useful for *in vitro* transformation assays of oncogenes, induced by the activation of endogenous β-catenin using a soluble form of Wnt-3a protein. Second, to confirm the role of the activation of β-catenin in the observed phenotypic alterations induced by Wnt-3a, we examined whether these changes were reproduced by the ectopic expression of a mutant β-catenin gene using an adenovirus vector.

MATERIAL AND METHODS

Conditioned medium containing soluble Wnt-3a protein

One million Wnt-3a-producing L cells, which were cloned from L cells transfected with pGKWnt-3a as described previously,[29] were seeded in a 100 mm dish containing DMEM (Nissui, Tokyo, Japan) with 10% FCS and L-gulutamine. After 3 days of culture, cells were refed with fresh medium and incubated for 1 more day and the cultured medium was collected as Wnt-3a-conditioned medium (W3a-CM). The medium was centrifuged at 1,000*g* for 10 min and filtered through a nitrocellulose membrane. As a control, conditioned medium from L cells transfected only with pGKneo and cultured under the same conditions as above was also prepared (L-CM).

Cell culture and transfection

NIH3T3, Balb3T3, Swiss3T3 and Rat-1 cells were cultured in DMEM with 10% FCS and L-glutamine. To measure the growth, death and saturation density of NIH3T3, Balb3T3, Swiss3T3 and Rat-1 cells, aliquots of 1×10^5 cells or 1×10^4 cells were seeded in 12-well plates. At several times after adding W3a-CM or L-CM, the cells were dissociated by trypsinization and counted. In the experiments performed in low-serum medium, viable cells were counted after trypan blue staining. At least 3 independent experiments were performed.

For plasmid transfection into the cells, we used the FuGENE6 transfection reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol.

Plasmid construction

The reporter plasmid p-55Bluc, which has an interferon-β (IFN-β) basal promoter followed by a luciferase gene, was kindly provided by Dr. Tohru Kiyono.[30] pTcf7wt-luc and pTcf7mt-luc were constructed by inserting the *Mlu*I-*Xho*I fragment of PCR products amplified using the oligonucleotides 5′-GACACGCGTCCTTTGATCCGCCCTTTGATCCTACCTTTGATCG-3′ and 5′-ACTCTCGAGGATCAAAGGCGCGATCAAAGGTAGGATCAAAGGG-3′ for pTcf7wt-luc; and 5′-GACACGCGTCCTTTGGCCCGCCCTTTGGCCCTACCTTTGGCCG-3′ and 5′-ACTCTCGAGGGCCAAAGGCGCGGCCAAAGGTAGGGCCAAAGGG-3′ for pTcf7mt-luc, into the *Mlu*I-*Xho*I site of p-55Bluc. Plasmid pcDNA3-Myc-ΔNTcf-4 was generated by inserting the *Bam*HI-*Xho*I fragment of the PCR product amplified using pcDNA-hTcf-4 (kindly supplied by Dr. Hans Clevers, University Hospital, Heidelberglaan, Utrecht, The Netherlands) and oligonucleotides 5′-CGAGGATCCATGAGCTCCGAAAACTCCTCGGC-3′ and 5′-ACTCTCGAGCGGGGTTCACGACGCTAAAG-3′ (R1), as template and primers, respectively, into the *Bam*HI-*Xho*I site of pcDNA3-Myc, which has been described previously.

Luciferase assay

Cells were transfected with reporter plasmid, pTcf7wt-luc or pTcf7mt-luc. Eight hours after transfection, the cell culture medium was removed and replaced with medium containing 20% W3a-CM or L-CM. After 20 hr of incubation with the medium, cells were lysed and the luciferase activity was quantified with a luciferase assay kit (Promega, Madison, WI) as recommended by the manufacturer.

Immunoblotting analysis

The preparation of cell lysates, SDS-PAGE and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as described previously.[31] The antibodies used in our experiment were anti-cyclin D1 (Ab-3; Calbiochem, San Diego, CA), anti-cyclin A (C-19), anti-cyclin E (M-20), anti-cdk2 (M2), anti-cdk4 (C-22) and anti-c-myc (9E10) (all from Santa Cruz Biotechnology, Santa Cruz, CA), as well as anti-α-tubulin (Ab-1; Calbiochem) antibodies. Immunocomplexes on the filters were detected by the enhanced chemiluminescence assay (Renaissance; NEN, Boston, MA).

Cell death detection ELISA

We utilized the Cell Death Detection ELISA kit (Boehringer Mannheim) according to the manufacturer's protocol. The assay is based on the specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of apoptotic cells. Briefly, after NIH3T3 cells were cultured in low serum medium for 9 hr, they were harvested and incubated in lysis buffer. The supernatant (cytoplasmic fraction) obtained after centrifuging the lysate was prepared as the sample solution for the following experiment. After coating the wells of a microtiter plate with anti-histone antibody, the sample solutions were added to the wells and incubated. After the wells were washed, they were incubated with anti-DNA antibody conjugated with peroxidase. After the wells were washed again, the substrate solution was added and the absorbance of the samples at 405 nm was measured.

Construction of recombinant adenovirus and infection of cells

For the construction of a recombinant adenovirus expressing LacZ and β-catenin SSTS/AAAA, in which the 4 amino-terminal GSK-3β phosphorylation sites (serine-33, -37 and -45, threonine-41) are changed to alanines, we used the Adeno-X™ expression system (ClonTech, Palo Alto, CA) according to the manufacturer's instructions. β-Catenin SSTS/AAAA cDNA was generated by PCR-based site-directed mutagenesis using pcDNA-β-catenin (kindly supplied by Dr. Hans Clevers, University Hospital, Heidelberglaan, Utrecht, The Netherlands) and oligonucleotides 5′-ATGGCTACTCAAGCTGATTTG-3′, 5′-TTACAGGTCAGTATCAAACCAGGCC-3′, 5′-GACGCTGGAATCCATGCTGGTGCCACTGCCACAGCTCCTGCTCTGAGTGGTAAAGGC-3′ and 5′-CAGAGCAGGAGCTGTGGCAGTGGCACCAGCATGGATTCCAGCGTCCAGGTAAGACTG-3′, as template and primers, respectively. We infected NIH3T3 cells and Rat-1 cells by incubation with virus-containing medium at 37°C for 1 hr, with brief agitation every 20 min. We then removed the virus-containing medium, added fresh medium and returned the cells to the 37°C incubator.

RESULTS

Transcriptional activation of β-catenin/Tcf-responsive promoter in rodent fibroblasts by addition of conditioned medium containing soluble Wnt-3a protein (W3a-CM)

To investigate the transcriptional activation of a β-catenin/Tcf-responsive promoter by W3a-CM in cultured cells, 2 reporter plasmids were constructed, as shown in Figure 1*a*. Reporter plasmid pTcf7wt-luc carries 7 repeats of the Tcf-binding consensus sequence upstream of the IFN-β basal promoter followed by the luciferase gene and pTcf7mt-luc has 7 repeats of a mutated Tcf-binding sequence instead of the wild-type sequence in pTcf7wt-luc. Rat-1 cells were transfected with each of these plasmids and then W3a-CM was added to the culture medium 8 hr after transfection. Luciferase activity in lysates prepared 20 hr after transfection was measured to assay the activity of Wnt/β-catenin signaling. As shown in Figure 1*b*, high luciferase activity from pTcf7wt-luc was induced by W3a-CM and the increase in the level of activity depended on the dose of W3a-CM, whereas the activity from pTcf7mt-luc was not affected by W3a-CM. W3a-CM did not induce an increase in luciferase activity in cells transiently expressing ΔNTcf-4, which is known to be a dominant negative type of Tcf-4[32] (Fig. 1*c*).

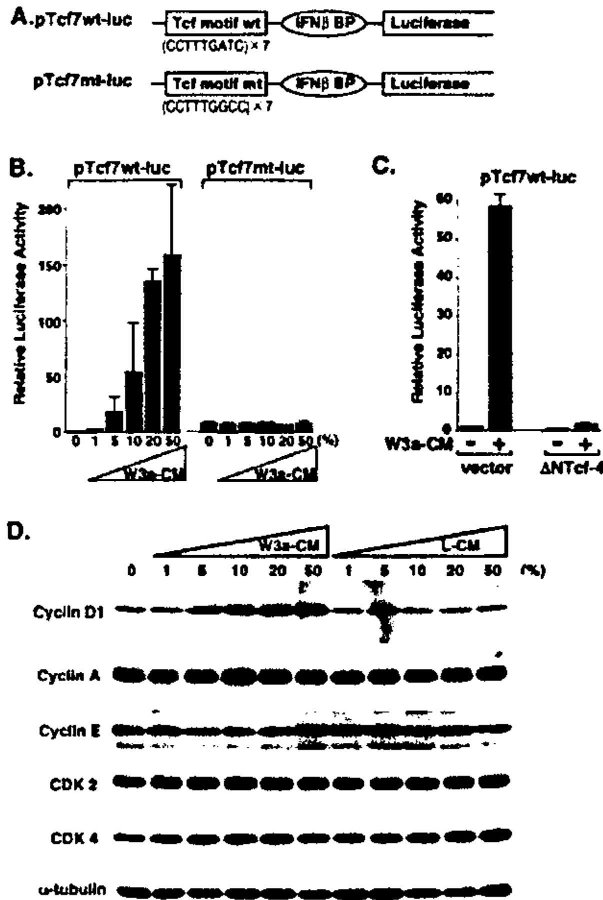

FIGURE 1 – Transcriptional activation of β-catenin/Tcf-responsive promoter by W3a-CM in rodent fibroblasts. (*a*) Schematic representation of reporter plasmids. (*b*) Activation of promoter containing Tcf-binding motifs by W3a-CM in Rat-1 cells. Rat-1 cells were transfected with 0.5 μg of pTcf7wt-luc or pTcf7mt-luc and then treated with various amounts of W3a-CM. The amount of W3a-CM relative to the total amount of culture medium is shown. Fold-activation was quantified relative to the level of luciferase activity in cells transfected with pTcf7wt-luc without treatment with W3a-CM. The values in the luciferase assay represent the means of 3 independent experiments. Error bars represent the standard error. (*c*) ΔNTcf-4 inhibits the transcriptional activation by W3a-CM. Rat-1 cells were transfected with 0.1 μg of pTcf7wt-luc and 0.5 μg of either pcDNA3 vector or pcDNA3-ΔNTcf-4 and then treated with medium containing 20% W3a-CM. Fold-activation was quantified relative to the level of luciferase activity in cells transfected with pTcf7wt-luc and pcDNA3 vector without treatment with W3a-CM. The data were analyzed essentially as described for (*b*) (*d*) W3a-CM induces endogenous cyclin D1 gene expression. Whole cell extracts of NIH3T3 cells treated with the indicated amounts of W3a-CM or L-CM were analyzed by immunoblot analysis for detection of endogenous proteins as indicated on the left.

These results indicated that W3a-CM induced transcriptional activity from a β-catenin/Tcf-responsive promoter through the β-catenin/Tcf complex in Rat-1 cells. In NIH3T3, Swiss3T3 and Balb3T3 cells, the transcriptional activation of this β-catenin/Tcf-responsive promoter by W3a-CM was observed in this reporter assay system, although the luciferase activity was weaker than that in Rat-1 cells (data not shown). Next, to examine whether the transcriptional activation of endogenous gene expression by W3a-CM is also observed, we investigated the levels of production of cyclin D1 protein in NIH3T3 cells by immunoblotting analysis. Cyclin D1 is known to be 1 of the target genes of β-catenin/Tcf.[14] As shown in Figure 1*d*, the level of cyclin D1 was increased in cells treated with W3a-CM in a dose-dependent manner, whereas the levels of cyclin A, cyclin E, cdk 2, cdk 4 and α-tubulin were not affected. These results indicated that Wnt-3a transcriptionally activated cyclin D1 expression through activation of β-catenin/Tcf. In conclusion, treatment with W3a-CM induced transcriptional activation of β-catenin/Tcf-responsive genes in these rodent fibroblasts.

W3a-CM induced an increase of saturation density in NIH3T3 cells

NIH3T3 cells are immortalized but retain the contact-inhibited growth phenotype and are known to be useful for oncogene transformation assays. To analyze the effect of W3a-CM on the growth and morphology of NIH3T3 cells, we cultured these cells in medium containing 20% W3a-CM. Three days after the addition of W3a-CM, an increase in cell density was observed, as shown in Figure 2*a*. Figure 2*b* shows the growth curves of NIH3T3 cells grown in DMEM with 10% FCS containing W3a-CM (W+FCS) or L-CM (L+FCS), or DMEM with 10% FCS only (+FCS). The number of NIH3T3 cells in W+FCS reached about 11 × 10^5 cells per well of a 12-well dish after 4 days of treatment with W3a-CM, whereas cells in either L+FCS or +FCS reached confluence at day 3 and stopped dividing at about 5 × 10^5 cells per well. These results showed that W3a-CM induces an increase of saturation density in NIH3T3 cells.

Next, we examined the effect of W3a-CM on the growth of NIH3T3 cells before they reached confluence. NIH3T3 cells were seeded sparsely (1 × 10^4 cells per well of a 12-well dish) and the cells were enumerated every 24 hr after the addition of W3a-CM or L-CM. As shown in Figure 3, no difference was seen between the cell growth in W+FCS and that in L+FCS before the cells reached confluence on day 5. This result was also confirmed by XTT-based colorimetric assay for quantification of cell proliferation using Cell Proliferation Kit II (XTT; Roche, Basel, Switzerland) (data not shown). These findings suggested that W3a-CM has no effect on cell growth before confluence.

W3a-CM suppresses apoptosis of NIH3T3 cells in low-serum medium

To investigate the effects of W3a-CM on the growth and death of NIH3T3 cells under low-serum conditions, we counted viable cells after shifting the cells to reduced serum-containing medium supplemented with 10% W3a-CM (W-FCS) or L-CM (L-FCS). The final concentration of FCS in the culture medium was 1% since the conditioned medium contained 10% FCS. It has been shown that NIH3T3 cells die of apoptosis in medium containing a

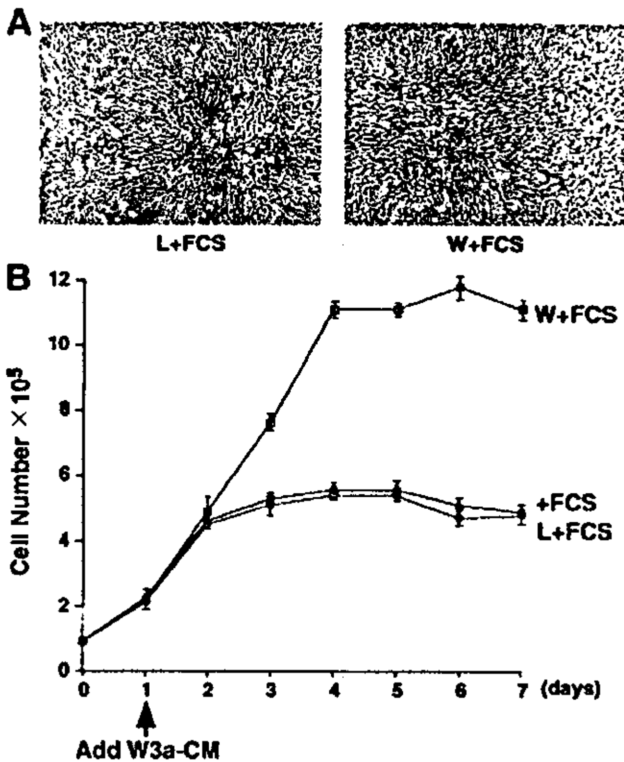

FIGURE 2 – W3a-CM induced an increase in saturation density of NIH3T3 cells. (*a*) Phase contrast microscopy of NIH3T3 cells cultured in medium containing 20% L-CM (L+FCS) or W3a-CM (W+FCS) for 3 days shows the difference in cell density. (*b*) Growth curve of NIH3T3 cells in culture medium with 10% serum containing 20% W3a-CM (W+FCS), 20% L-CM (L+FCS) or no addition (+FCS). Aliquots of 1×10^5 cells were inoculated in 12-well plates and 24 hr after passage, the culture medium was replaced by W+FCS, L+FCS, or +FCS. Cells numbers are presented as means of the numbers in 3 independent plates. Error bars represent the standard error. Squares, W+FCS; circles, L+FCS; triangles, +FCS.

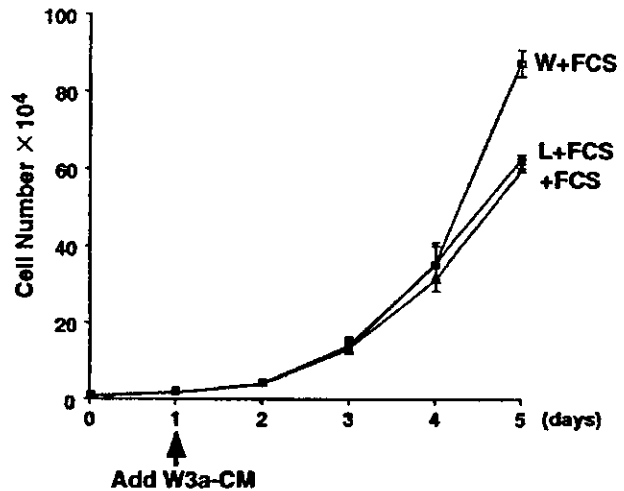

FIGURE 3 – W3a-CM has no effect on the growth of NIH3T3 cells before they reach confluence. NIH3T3 cells were seeded at low density (1×10^4 cells/12-well plate) and 24 hr after passage, the culture medium was replaced by W+FCS, L+FCS or +FCS. The data were analyzed essentially as described for Figure 2*b*. Squares, W+FCS; circles, L+FCS; triangles, +FCS.

low level of serum.[33,34] However, the number of viable cells in W-FCS did not change until 48 hr after the shift to the reduced serum medium, whereas that in L-FCS decreased and reached about 30% of the original cell number (Fig. 4*a*,*b*). To confirm that the effect of W3a-CM reflects inhibition of apoptosis, we performed a colorimetric apoptosis assay in which the amount of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of apoptosis-induced cells can be determined by measuring the absorbance. As shown in Figure 4*c*, apoptosis was inhibited in cells cultured in W-FCS compared with those cultured in L-FCS. In summary, the addition of W3a-CM to NIH3T3 cells in low-serum medium suppresses apoptosis in these cells.

Removal of W3a-CM reverses the increase in saturation density and induces apoptosis in low-serum medium in NIH3T3 cells

To examine whether continuous stimulation by W3a-CM is required for the increase in saturation density and the inhibition of apoptosis, these effects on NIH3T3 cells were analyzed. The increased saturation density of cells cultured in W+FCS was gradually reduced by removal of W3a-CM (Fig. 5*a*) and eventually reached almost the same level as that of cells grown in L+FCS. Likewise, in low-serum conditions, removal of W3a-CM induced a decrease in the viable cell number (Fig. 5*b*). These results showed that the increase in saturation density and inhibition of apoptosis in low serum-medium is dependent on the continuous presence of W3a-CM.

Effects of W3a-CM on Rat-1, Balb3T3 and Swiss3T3 cells

To investigate whether these effects of W3a-CM are specific to NIH3T3 cells, other rodent fibroblast cell lines, Rat-1, Swiss3T3 and Balb3T3 cells, were examined by culturing in medium containing 20% W3a-CM. As shown in Figure 6*a*, W3a-CM also induced an increase of saturation density in all these cells. Furthermore, in low-serum medium, the viable cell numbers of these lines in W-FCS at 48 hr after serum removal were higher than those in L-FCS or 1% FCS medium (Fig. 6*b*). As the death of Rat-1 cells was not induced by serum starvation of L-FCS or 1% FCS, the increase in the viable number of cells in this line in W-FCS seems likely to have been caused by the promotion of serum-independent growth by W3a-CM. Similarly, the increase in viable Balb3T3 and Swiss3T3 cells in W-FCS compared with L-FCS and 1% FCS might have been caused by both anti-apoptosis and serum-independent growth effects of W3a-CM, because the viable cell numbers at 48 hr in W-FCS were greater than those before serum depletion. Moreover, Rat-1 cells became elongated and refractile in response to the addition of W3a-CM, as shown in Figure 6*c*. These morphologic changes could be reversed by removal of W3a-CM (Fig. 6*c*).

These changes caused by W3a-CM were observed even when the cells were seeded sparsely (data not shown), indicating that the cell-cell interactions are not required for the morphologic changes to Rat-1 cells. In summary, W3a-CM induced phenotypical alterations in these rodent fibroblasts: an increase in saturation density, morphologic changes and the inhibition of apoptosis and/or promotion of growth in low-serum medium.

Expression of mutant β-catenin induces morphologic change and suppresses apoptosis

To investigate whether the observed phenotypical changes induced by W3a-CM are due to the activation of β-catenin, we analyzed the effects of the ectopic expression of mutant β-catenin on NIH3T3 and Rat-1 cells. A myc-epitopic-tagged mutant β-catenin gene (β-catenin SSTS/AAAA) was obtained by site-directed mutation of 3 serine residues and 1 threonine residue, all phosphorylation sites of GSK-3β, at positions 33, 37, 45 and 41, respectively, to alanines.[6] β-Catenin SSTS/AAAA is expected to be resistant to degradation by proteasomes and thus to accumulate in cells. This was confirmed by immunoblot analysis and reporter

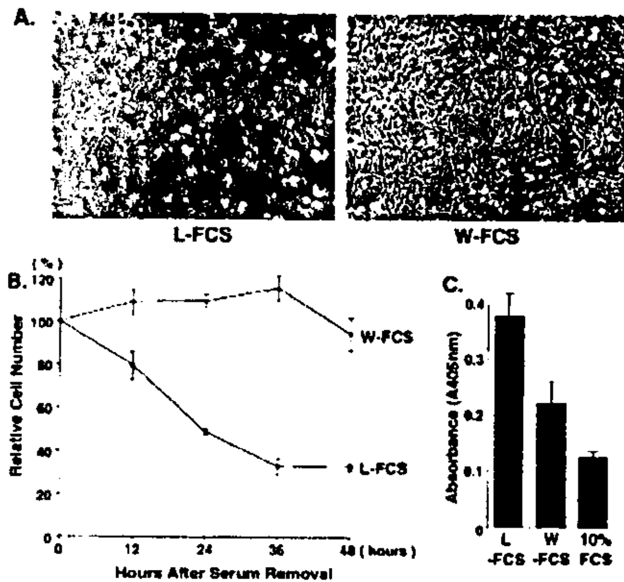

FIGURE 4 – W3a-CM suppresses apoptosis of NIH3T3 cells in low-serum medium. (*a*) Phase contrast microscopy of NIH3T3 cells cultured in serum-free medium containing 10% L-CM (L-FCS) or W3a-CM (W-FCS) at 48 hr after serum removal shows the difference in viable cell numbers. (*b*) Time course of the change in the relative numbers of viable NIH3T3 cells in serum-free medium containing 10% W3a-CM (W-FCS) or 10% L-CM (L-FCS). Aliquots of 1×10^5 cells were inoculated in 12-well plates and 24 hr after passage, the culture medium was replaced by W-FCS or L-FCS. The relative cell number was calculated as a percentage relative to the cell number recorded before serum removal. Cell numbers are presented as means of the values for 3 independent plates. Error bars represent the standard error. (*c*) Apoptosis assay in NIH3T3 cells at 9 hr after serum removal using a cell death detection ELISA kit (Boehringer Mannheim) shows inhibition of apoptosis of NIH3T3 cells in W-FCS compared with L-FCS. NIH3T3 cells in culture medium containing 10% FCS were used as a negative control in this assay and are indicated as 10% FCS. The absorbance at 405 nm represents the average of 3 independent experiments. Error bars represent the standard error.

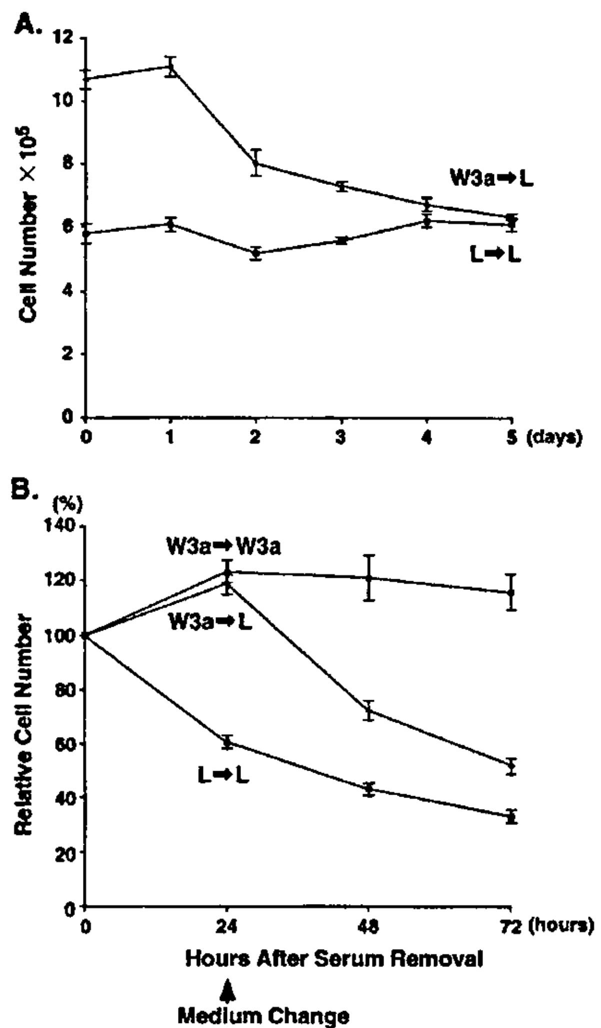

FIGURE 5 – Removal of W3a-CM reverses the increase in saturation density and induces apoptosis in low-serum medium in NIH3T3 cells. (*a*) Time course of the change in NIH3T3 cell numbers after removal of W3a-CM or L-CM. After 4 days of culture in W+FCS or L+FCS, the culture medium was changed to L+FCS at day 0 in the graph. W3a→L indicates a medium change from W+FCS to L+FCS and L→L that from L+FCS to L+FCS. The data were analyzed essentially as described for Figure 2*b*. (*b*) Time course of the change in the relative number of viable NIH3T3 cells in serum-free medium containing 10% W3a-CM (W-FCS) or 10% L-CM (L-FCS). Aliquots of 1×10^5 cells were inoculated in 12-well plates and 24 hr after passage, the culture medium was changed to W-FCS or L-FCS. After 24 hr in W-FCS or L-FCS, the culture medium was changed again as indicated. W3a→W3a and W3a→L indicate a medium change from W-FCS to W-FCS and L-FCS, respectively and L→L indicates that from L-FCS to L-FCS. The data were analyzed essentially as described for Figure 4*b*.

gene assays showing that the protein level and transcriptional activity of β-catenin SSTS/AAAA were higher than those of wild-type β-catenin in Saos-2 cells in transient transfection experiments (data not shown). In order to express the β-catenin SSTS/AAAA gene uniformly in as many cells as possible, we used a recombinant adenovirus expressing the myc-tagged mutant β-catenin gene (Ad-β-cat) and a recombinant adenovirus expressing the β-galactosidase gene (Ad-LacZ) as a negative control vector.

β-Galactosidase staining after infection by Ad-LacZ at a multiplicity of infection (MOI) of 100 showed that 80–90% of NIH3T3 cells were infected (data not shown). We also confirmed that in the lysate of the cells infected by Ad-β-cat at an MOI of 100, cyclin D1 protein was increased (Fig. 7*a*, lane 4) to a level as high as that in the lysate of cells cultured in W3a-CM-containing medium (Fig. 7*a*, lane 3). Induction of apoptosis was not observed in the cells expressing β-catenin SSTS/AAAA in our experiments, unlike in previous reports.[18] Using the above conditions of infection, we investigated the changes in saturation density and apoptosis in low-serum medium for NIH3T3 cells infected by Ad-β-cat. The saturation density of the cells was not increased by infection with Ad-β-cat (Fig. 7*b*, lane 3), although the infected cells have the potential to increase their saturation density in response to treatment with W3a-CM (Fig. 7*b*, lane 4). In contrast, the viable number of NIH3T3 cells infected by Ad-β-cat was elevated even in reduced serum medium (Fig. 7*c*, lane 4) and the anti-apoptotic effect was the same as that of W3a-CM-treated NIH3T3 cells that were infected by Ad-LacZ (Fig. 7*c*, lane 3). The increase in viable cell number caused by the addition of W3a-CM to Ad-β-cat-infected cells (Fig. 7*c*, lanes 4,5) was thought to be caused by an anti-apoptotic effect on noninfected cells, because the efficiency of infection was about 80–90% in NIH3T3 cells.

In Rat-1 cells, 100% of cells exhibited β-galactosidase staining after infection with Ad-LacZ at an MOI of 10–100 (data not shown). The transcriptional activation of the reporter gene driven by a β-catenin/Tcf-responsive promoter was observed in Rat-1 cells infected by Ad-β-cat (Fig. 7*d*). The luciferase activity in Rat-1 cells infected with Ad-β-cat at an MOI of 50 was as high as

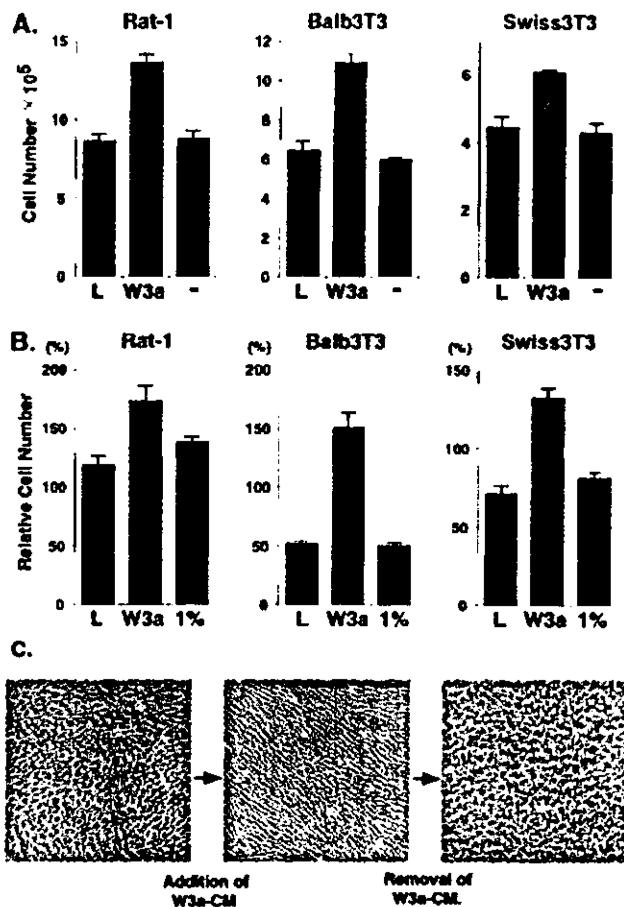

FIGURE 6 – Effects of W3a-CM on Rat-1, Balb3T3 and Swiss3T3 cells. (*a*) Increase of saturation density in Rat-1, Balb3T3 and Swiss3T3 cells by W3a-CM. The numbers of Rat-1, Balb3T3 and Swiss3T3 cells after 4 days in L+FCS (L), W+FCS (W3a) or +FCS (-) are shown. (*b*) Inhibition of cell death and/or promotion of serum-independent growth in Rat-1, Balb3T3 and Swiss3T3 cells by W3a-CM. The relative numbers of Rat-1, Balb3T3 and Swiss3T3 cells at 48 hr after serum removal were calculated as a percentage relative to the cell number before serum removal. L, W3a and 1% indicate the cells in W-FCS, L-FCS and medium containing 1% serum, respectively. The cell numbers in (*a*) and (*b*) are the averages of 3 independent experiments. Error bars represent the standard error. (*c*) Morphologic change in Rat-1 cells caused by addition and then removal of W3a-CM. Rat-1 cells were seeded at high density (3×10^5 cells/well in 12-well dishes) and the culture medium was changed to W+FCS at 48 hr after passage and again to +FCS after 48 hr in W+FCS. The left panel shows the morphology of cells 48 hr after passage, the middle panel that at 48 hr in W+FCS and the right panel that at 4 days after removal of W3a-CM.

that in the cells treated with W3a-CM (Fig. 7*d*, lanes 3,4). As shown in Figure 7*e*, the morphology of Rat-1 cells infected with Ad-β-cat at an MOI of 50 was altered to resemble that of cells treated with W3a-CM. However, the saturation density of Rat-1 cells was not increased by infection with Ad-β-cat (data not shown).

In conclusion, the expression of mutant β-catenin induced a suppression of cell death in NIH3T3 cells and morphologic changes in Rat-1 cells, effects also induced by treatment with W3a-CM but did not cause an increase of saturation density in these cells.

DISCUSSION

We have shown here that W3a-CM induced 3 major phenotypic alterations in rodent fibroblasts: an increase in saturation density, morphologic change and inhibition of apoptosis and/or promotion of growth in low-serum medium. In the mammary epithelial cell lines C57MG and RAC311C,[35,36] Wnt-1 expression resulting from stable transfection causes morphologic changes and postconfluent growth. It was also reported that the soluble form of Wnt-1 protein induced morphologic changes and postconfluent growth by acting as a diffusible extracellular signaling factor in mammary epithelial cells.[37] Similarly, in fibroblasts, serum-independent cellular proliferation and morphologic changes were reported in Wnt-1-transfected Rat-1 cells,[21] as well as increases of saturation density and growth in soft agar in Wnt-1 and -2-transfected NIH3T3 cells.[17] Our results suggest that Wnt-3a shares some function(s) with Wnt-1 and Wnt-2 and that the changes in fibroblasts were caused by Wnt protein acting as a diffusible extracellular signaling factor.

In addition, we showed an anti-apoptotic effect of Wnt-3a in low-serum medium. Anti-apoptotic effects were also induced in NIH3T3 cells by infection with Ad-β-cat. This finding suggested that activation of β-catenin alone is sufficient to elicit anti-apoptotic effects in NIH3T3 cells. There is a significant relationship between apoptosis and cancer. Acquired resistance to apoptosis is thought to be associated with the carcinogenesis of most and perhaps all types of cancer.[38,39] In fact, many oncogenes, tumor suppressor genes and proteins produced by oncogenetic viruses have pro- or anti-apoptotic effects.[40] The anti-apoptotic effect of β-catenin, which we showed here, may be 1 feature of carcinogenesis.

We showed that morphologic changes in Rat-1 cells were also induced by infection with Ad-β-cat. However, Young *et al.*[21] reported that expression of a mutant β-catenin in Rat-1 cells did not result in any detectable morphologic change. The reason for the difference in the results is not clear, but it may be due to the quantity of β-catenin. Infection by adenovirus vector can induce the expression of recombinant proteins encoded by the virus and the expression level of these proteins can be altered by altering the MOI. Indeed, no morphologic change was induced by infection of Ad-β-cat at an MOI of 20, under which conditions the level of expression of β-catenin protein and the transcriptional activity of the β-catenin/Tcf-responsive promoter were much lower than at an MOI of 50, as shown by immunoblot analysis (data not shown).

No morphologic change caused by W3a-CM was detected in NIH3T3, Balb3T3 and Swiss3T3 cells, suggesting that the response to Wnt is different between cell types. This finding indicates that the downstream area of Wnt signaling other than β-catenin signaling or the target gene of β-catenin/Tcf differs between cell types. It was recently reported that *Drosophila* Rho-associated kinase (Drok) works downstream of Frizzled to mediate planer cell polarity.[41] The mammalian homolog of Drok might have induced the morphologic change shown in this paper.

No increase in saturation density was induced by the ectopic expression of mutant β-catenin encoded by an adenovirus vector. This finding suggests that the expression of β-catenin alone is not sufficient to increase saturation density. Recent reports suggest that the activation of β-catenin is 1 of several downstream events in the Wnt signal transduction pathway. Other signaling pathways activated by Wnt have been reported; 1 is the Wnt/Ca^{2+} pathway, which stimulates an increase in intracellular Ca^{2+} and the subsequent activation of protein kinase C[42–44] and another is the c-Jun N-terminal kinase/stress-activated protein kinase pathway, which was reported to be activated by dishevelled, a well-known transducer of Wnt signaling, in a process that is independent of its effects on the stabilization of β-catenin.[45] These signaling pathways may be essential to the increase in saturation density.

The alterations induced by W3a-CM and mutant β-catenin in rodent fibroblasts are expected to be useful markers for analysis of the mechanisms of the phenotypic changes caused by the activation of Wnt/β-catenin signaling. Further investigation of these mechanisms should increase our understanding of tumorigenesis.

During the preparation of our paper, Chen *et al.*[46] reported that Wnt-1 signaling inhibited apoptosis by activating β-catenin/Tcf-

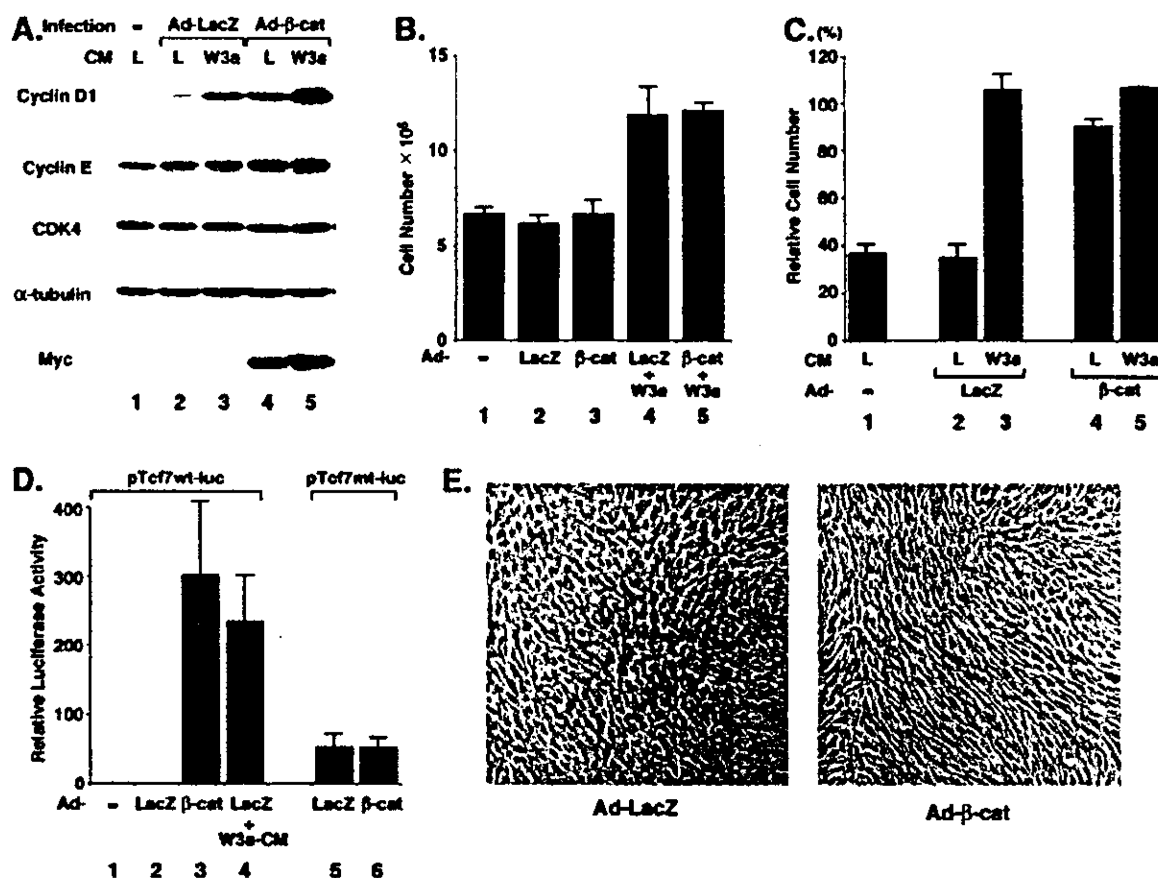

FIGURE 7 – Expression of mutant β-catenin induces morphologic change and suppresses apoptosis. (*a*) Increase of endogenous cyclin D1 protein in NIH3T3 cells infected with Ad-β-cat. NIH3T3 cells were infected with Ad-LacZ or Ad-β-cat at a MOI of 100; at 24 hr after infection, the culture medium was changed to L+FCS or W+FCS and total cell lysates were prepared at 24 hr after the addition of L-CM or W3a-CM were analyzed by immunoblotting using the antibodies indicated on the left. (*b*) Lack of increase in saturation density of NIH3T3 cells infected with Ad-β-cat. NIH3T3 cells were infected with Ad-LacZ or Ad-β-cat at an MOI of 100 and cultured for 4 days in medium containing 10% FCS with or without W3a-CM. Cell numbers were counted 4 days after infection and are expressed as the means of 3 plates. Error bars represent the standard error. (*c*) Inhibition of cell death in NIH3T3 cells infected with Ad-β-cat. NIH3T3 cells were infected with Ad-LacZ or Ad-β-cat at an MOI of 100 and at 24 hr after infection the culture medium was changed to L-FCS or W-FCS. Viable cells were enumerated at 48 hr after serum removal. The data were analyzed essentially as described for Figure 6*b*. (*d*) Activation of promoter-containing Tcf-binding motifs by infection with Ad-β-cat in Rat-1 cells. Fold-activation was quantified relative to the level of luciferase activity in the control cells, which were not infected. The data of the luciferase assay were analyzed essentially as described for Figure 1. (*e*) Morphologic changes induced by Ad-β-cat in Rat-1 cells. The morphology of Rat-1 cells at 48 hr after infection with Ad-LacZ (left panel) and Ad-β-cat (right panel) at an MOI of 50 is shown.

mediated transcription. They showed that inhibition of β-catenin/Tcf transcription by expression of the dominant-negative mutant of Tcf-4 blocked Wnt-1-mediated cell survival. This result indicates that activation of β-catenin/Tcf-mediated transcription is essential to the Wnt-1-mediated anti-apoptotic effect, but it was not clear whether β-catenin alone is sufficient for the inhibition of apoptosis. Their result may support our findings that expression of β-catenin induced inhibition of apoptosis. Our results provide direct evidence that the inhibition of apoptosis is induced by the expression of β-catenin.

REFERENCES

1. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 1997;11:3286–305.
2. Peifer M, Polakis P. Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science 2000;287:1606–9.
3. Kikuchi A. Regulation of β-catenin signaling in the Wnt pathway. Biochem Biophys Res Commun 2000;268:243–8.
4. Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling [In Process Citation]. Cell 2000;103:311–20.
5. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
6. Morin PJ. β-catenin signaling and cancer. Bioessays 1999;21:1021–30.
7. Polakis P. Wnt signaling and cancer. Genes Dev 2000;14:1837–51.
8. Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM. Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. EMBO J 1999;18:5931–42.
9. Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc Natl Acad Sci USA 1995;92:4482–6.
10. Whitehead I, Kirk H, Kay R. Expression cloning of oncogenes by retroviral transfer of cDNA libraries. Mol Cell Biol 1995;15:704–10.
11. Kolligs FT, Hu G, Dang CV, Fearon ER. Neoplastic transformation of RK3E by mutant β-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. Mol Cell Biol 1999;19:5696–706.
12. Barth AI, Stewart DB, Nelson WJ. T cell factor-activated transcription is not sufficient to induce anchorage-independent growth of epithelial

cells expressing mutant β-catenin. Proc Natl Acad Sci USA 1999;96: 4947–52.
13. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–12.
14. Tetsu O, McCormick F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
15. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and β-catenin-responsive oncogene. Genes Dev 2000;14:585–95.
16. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 1999;99:335–45.
17. Bafico A, Gazit A, Wu-Morgan SS, Yaniv A, Aaronson SA. Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. Oncogene 1998;16:2819–25.
18. Kim K, Pang KM, Evans M, Hay ED. Overexpression of β-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol Biol Cell 2000;11:3509–23.
19. Nagasawa Y, Miyoshi Y, Iwao K, Shinomura Y, Matsuzawa Y, Nakamura Y. Transformation and morphological changes of murine L cells by transfection with a mutated form of β-catenin. Cancer Res 1999;59:3539–42.
20. Orford K, Orford CC, Byers SW. Exogenous expression of β-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. J Cell Biol 1999;146:855–68.
21. Young CS, Kitamura M, Hardy S, Kitajewski J. Wnt-1 induces growth, cytosolic β-catenin and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. Mol Cell Biol 1998;18:2474–85.
22. Ahmed Y, Hayashi S, Levine A, Wieschaus E. Regulation of armadillo by a *Drosophila* APC inhibits neuronal apoptosis during retinal development. Cell 1998;93:1171–82.
23. Zhang Z, Hartmann H, Do VM, Abramowski D, Sturchler-Pierrat C, Staufenbiel M, Sommer B, van de Wetering M, Clevers H, Saftig P, De Strooper B, He X, et al. Destabilization of β-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. Nature 1998;395:698–702.
24. Morin PJ, Vogelstein B, Kinzler KW. Apoptosis and APC in colorectal tumorigenesis. Proc Natl Acad Sci USA 1996;93:7950–4.
25. Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, et al. AXIN1 mutations in hepatocellular carcinomas and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet 2000;24:245–50.
26. Bournat JC, Brown AM, Soler AP. Wnt-1 dependent activation of the survival factor NF-kappaB in PC12 cells. J Neurosci Res 2000;61: 21–32.
27. Bradbury JM, Niemeyer CC, Dale TC, Edwards PA. Alterations of the growth characteristics of the fibroblast cell line C3H 10T1/2 by members of the Wnt gene family. Oncogene 1994;9:2597–603.
28. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells [see comments]. Nat Genet 2000;24:372–6.
29. Shibamoto S, Higano K, Takada R, Ito F, Takeichi M, Takada S. Cytoskeletal reorganization by soluble Wnt-3a protein signalling. Genes Cells 1998;3:659–70.
30. Kiyono T, Hiraiwa A, Ishii S, Takahashi T, Ishibashi M. Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. J Virol 1994;68:4656–61.
31. Ueda Y, Hijikata M, Takagi S, Chiba T, Shimotohno K. New p73 variants with altered C-terminal structures have varied transcriptional activities. Oncogene 1999;18:4993–8.
32. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon. Science 1997;275: 1784–7.
33. Okamoto K, Prives C. A role of cyclin G in the process of apoptosis. Oncogene 1999;18:4606–15.
34. Schroter M, Peli J, Hahne M, Tschopp J, Reichmann E. Fas-dependent tissue turnover is implicated in tumor cell clearance. Oncogene 2000; 19:1794–800.
35. Brown AM, Wildin RS, Prendergast TJ, Varmus HE. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell 1986;46:1001–9.
36. Rijsewijk F, van Deemter L, Wagenaar E, Sonnenberg A, Nusse R. Transfection of the int-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J 1987;6:127–31.
37. Bradley RS, Brown AM. A soluble form of Wnt-1 protein with mitogenic activity on mammary epithelial cells. Mol Cell Biol 1995; 15:4616–22.
38. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100: 57–70.
39. Sellers WR, Fisher DE. Apoptosis and cancer drug targeting. J Clin Invest 1999;104:1655–61.
40. White E. Life, death and the pursuit of apoptosis. Genes Dev 1996; 10:1–15.
41. Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L. *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 2001;105: 81–91.
42. Miller JR, Hocking AM, Brown JD, Moon RT. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca^{2+} pathways. Oncogene 1999;18:7860–72.
43. Kuhl M, Sheldahl LC, Malbon CC, Moon RT. Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in *Xenopus*. J Biol Chem 2000;275:12701–11.
44. Chen RH, Ding WV, McCormick F. Wnt signaling to β-catenin involves two interactive components. Glycogen synthase kinase-3 b inhibition and activation of protein kinase C. J Biol Chem 2000;275: 17894–9.
45. Moriguchi T, Kawachi K, Kamakura S, Masuyama N, Yamanaka H, Matsumoto K, Kikuchi A, Nishida E. Distinct domains of mouse dishevelled are responsible for the c-Jun N-terminal kinase/stress-activated protein kinase activation and the axis formation in vertebrates. J Biol Chem 1999;274:30957–62.
46. Chen S, Guttridge DC, You Z, Zhang Z, Fribley A, Mayo MW, Kitajewski J, Wang CY. Wnt-1 signaling inhibits apoptosis by activating β-catenin/T cell factor-mediated transcription. J Cell Biol 2001;152:87–96.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 278, No. 12, Issue of March 21, pp. 10162–10173, 2003
Printed in U.S.A.

Identification of a Lactoferrin-derived Peptide Possessing Binding Activity to Hepatitis C Virus E2 Envelope Protein*

Received for publication, August 2, 2002, and in revised form, December 27, 2002
Published, JBC Papers in Press, January 9, 2003, DOI 10.1074/jbc.M207879200

Akito Nozaki‡, Masanori Ikeda§, Atsushi Naganuma‡¶, Takashi Nakamura‡, Michiharu Inudoh‖, Katsuaki Tanaka§, and Nobuyuki Kato‡**

From the ‡Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan, §Gastroenterological Center, Yokohama City University Medical Center, Yokohama City University School of Medicine, Yokohama 236-0004, Japan, and ‖Department of Pediatrics, Tsukuba University School of Medicine, Tsukuba 305-8575, Japan

Bovine and human lactoferrins (LF) prevent hepatitis C virus (HCV) infection in cultured human hepatocytes; the preventive mechanism is thought to be the direct interaction between LF and HCV. To clarify this hypothesis, we have characterized the binding activity of LF to HCV E2 envelope protein and have endeavored to determine which region(s) of LF are important for this binding activity. Several regions of human LF have been expressed and purified as thioredoxin-fused proteins in *Escherichia coli*. Far-Western blot analysis using these LF fragments and the E2 protein, expressed in Chinese hamster ovary cells, revealed that the 93 carboxyl amino acids of LF specifically bound to the E2 protein. The 93 carboxyl amino acids of LFs derived from bovine and horse cells also possessed similar binding activity to the E2 protein. In addition, the amino acid sequences of these carboxyl regions appeared to show partial homology to CD81, a candidate receptor for HCV, and the binding activity of these carboxyl regions was also comparable with that of CD81. Further deletion analysis identified 33 amino acid residues as the minimum binding site in the carboxyl region of LF, and the binding specificity of these 33 amino acids was also confirmed by using 33 maltose-binding protein-fused amino acids. Furthermore, we demonstrated that the 33 maltose-binding protein-fused amino acids prevented HCV infection in cultured human hepatocytes. In addition, the site-directed mutagenesis to an Ala residue in both terminal residues of the 33 amino acids revealed that Cys at amino acid 628 was determined to be critical for binding to the E2 protein. These results led us to consider the development of an effective anti-HCV peptide. This is the first identification of a natural protein-derived peptide that specifically binds to HCV E2 protein and prevents HCV infection.

Hepatitis C virus (HCV)[1] infection frequently causes chronic hepatitis (1, 2) and frequently progresses to liver cirrhosis and hepatocellular carcinoma (3, 4). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* (5–7). The HCV genome encodes a large polyprotein precursor of about 3,000 amino acid (aa) residues, which is cleaved by the host and viral proteases to generate at least ten proteins: the core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (8–12). The most characteristic feature of the HCV genome is its remarkable sequence heterogeneities and variations, and to date at least six major HCV genotypes, which have been further grouped into more than 50 subtypes, have been identified (13–16). The genetic complexity of HCV is thus a major hindrance to the development of the vaccines.

To date, interferon has been the sole effective antiviral reagent used in the clinical therapy of hepatitis C, but its effectiveness is limited to about 30% of the reported cases (17). Combined treatment of interferon and ribavirin has been shown to be more effective than treatment with interferon alone (18). The side effects of interferon are also in some cases severe enough to lead to treatment cessation.

Although the entry mechanism of HCV, as well as that of hepatitis B virus, remains unclear, it was reported recently that human CD81 (19) and scavenger receptor class B type I (20) could be bound by a truncated, soluble form of the E2 protein; such findings suggest that these proteins may act as receptors for HCV on the cell surface. Low density lipoprotein receptor (21) was also reported as a putative HCV receptor in endocytosis experiments using isolated HCV-lipoprotein complexes. However, because of the lack of a reproducible and efficient HCV proliferation system, it is not known whether these candidate receptors for HCV serve as the functional receptor on human hepatocytes (22).

We previously reported that non-neoplastic human hepatocyte-derived PH5CH8 cells supported HCV replication, although HCV proliferation was at a fairly low level; in that study, we also demonstrated the antiviral effects of interferon-α in HCV-infected PH5CH8 cells (23). Using a PH5CH8 cell culture system, we found that bovine and human lactoferrin (LF), a milk glycoprotein belonging to the iron transporter family, specifically prevented HCV infection in the cells (24). Recently, Matsuura *et al.* (25) also showed that bovine LF specifically inhibited infection by the pseudotype vesicular stomatitis virus possessing chimeric HCV E1 and E2 glycoproteins.

LF has a molecular mass of 80 kDa and consists of two

* This work was supported by grants-in-aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control and for research on hepatitis and BSE from the Ministry of Health, Labor and Welfare and by grants-in-aid for scientific research from the Organization for Pharmaceutical Safety and Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: The First Department of Internal Medicine, Gunma University School of Medicine, Maebashi 371-8511, Japan.

** To whom correspondence should be addressed. Tel.: 81-86-235-7385; Fax: 81-86-235-7392; E-mail: nkato@md.okayama-u.ac.jp.

[1] The abbreviations used are: HCV, hepatitis C virus; LF, lactoferrin; aa, amino acid; TF, transferrin; BSA, bovine serum albumin; PNGaseF, peptide-*N*-glycosidase F; RT, reverse transcription; LEL, large extracellular loop; TRX, thioredoxin; MBP, maltose-binding protein; ELISA, enzyme-linked immunosorbent assay; HBS, HEPES-buffered saline.

This paper is available on line at http://www.jbc.org