Functional Absence of FADD in PLC/PRF/5 Hepatoma Cells: Possible Involvement in the Transformation to Hepatoma in HBV-Infected Hepatocytes (44386)

ATSUSHI SUZUKI*,[1] TAKASHI ARAKI,† MASAYUKI MIURA,† AND YUMI TSUTOMI*

Drug Safety Research Laboratory, Daiichi Pharmaceutical Co., Ltd., Tokyo 134, Japan; and Department of Neuroanatomy,† Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan*

Abstract. **The death receptor Fas transduces apoptotic death signaling upon stimulation by Fas ligand and plays a key role in viral hepatitis. When hepatitis-B virus (HBV) infects hepatocytes, the Fas ligand/Fas system responds as the triggering machinery of hepatitis. However, some HBV-infected cells may circumvent Fas-mediated apoptosis and transform to hepatoma cells, as do PLC/PRF/5 hepatoma cells. Therefore, in the present study, we used PLC/PRF/5 hepatoma cells to investigate this ability to avoid Fas-mediated apoptosis. When the cells were treated with an agonistic Fas antibody, they showed resistance to Fas-mediated apoptosis. In contrast, HepG2 cells of the same hepatoma line succumbed. Caspase 3 and 8, which are essential regulators for Fas-mediated cell death, were expressed in both hepatoma cell lines, but only HepG2 cells showed activation of the caspases. A comparison study of expression of other death-associated factors between PLC/PRF/5 and HepG2 cells revealed no apparent differences. However, Far-Western blotting analysis using the Fas death domain (FDD) showed a significant difference. Molecular weight comparison and immunoblotting analysis revealed that PLC/PRF/5 cells lack the FDD-associated protein FADD. In addition, FDD-injected HepG2 cells showed a resistance to Fas-mediated apoptosis, and PLC/PRF/5 cells acquired Fas-sensitivity by FADD injection. Here, we propose that a functional absence of FADD is one of the pathways for the carcinogenesis of HBV-infected hepatocytes.** [P.S.E.B.M. 1999, Vol 221]

Programmed cell death is a physiological process indispensable for the development and homeostatic maintenance of multicellular organisms. Most programmed cell death follows the apoptotic pathway (1). Apoptotic cell death is accompanied by condensation and/or fragmentation of nuclei, loss of plasma membrane microvilli, condensation of cytoplasm, and fragmentation of chromosomal DNA into 180-bp oligomers (1). Apoptotic cell death has been encountered in embryonic and postembryonic development (1).

The death receptor Fas is a cell surface molecule belonging to the nerve growth factor/tumor necrosis factor receptor family, and it transduces the apoptotic death signaling upon stimulation by Fas ligand (2). Fas also plays a dominant role in physiological cell death and various disease states (2–5). Recently, the dominant role of Fas in hepatitis has been demonstrated (2, 6–9). When hepatocytes are infected with hepatitis virus, including hepatitis-B virus (HBV), viral antigen is expressed on the surface of the infected cell (10). Cytotoxic T lymphocytes (CTLs) recognize this antigen, and Fas ligand expressed on the CTL's surface binds to Fas upon contact of the CTL and the hepatitis virus–infected cell. Thus, CTLs remove the infected hepatocytes *via* induction of Fas-mediated apoptosis, namely CTL's kiss of death (11). While most hepatitis vi-

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[1] To whom requests for reprints should be addressed at Daiichi Pharmaceutical Co., Ltd., Drug Safety Research Laboratory, Tokyo R&D Center, 16-13 Kitakasai 1, Edogawa-ku,Tokyo 134, Japan. E-mail:LEB00373@niftyserve.or.jp

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rus–infected hepatocytes are removed by this mechanism, some infected cells do manage to avoid this process and ultimately transform to hepatoma.

The molecular mechanism of Fas-mediated apoptosis has been the subject of extensive investigation. Various genes have been identified that function to drive the death machinery and that act against apoptotic cell death. Fas contains multiple regions, one of which is the "death domain" (2, 12). The death domain is an essential region for the transduction of Fas-mediated death signaling (13). Recently, FADD has been identified as a Fas-death domain–associated protein (14) that mediates apoptotic death signaling by binding to the Fas-death domain. Some death mediators, such as caspase 8 (FLICE/MACH and Refs. 15 and 16), interact with the Fas-death domain *via* FADD. This complex formation (Fas-death domain, FADD, and caspase 8) is termed Fas-DISC (Death-Inducing Signaling Complex).

The human hepatoma PLC/PRF/5 cell line (17) is a hepatoma line that transforms due to its ability to avoid Fas-mediated apoptosis induced by HBV. This hepatoma carries HBV and expresses its cell surface antigen (18, 19). This avoidance of Fas-mediated apoptosis and carcinogenesis to hepatoma is an interesting phenomenon for the investigation of Fas-initiated death signaling. We report here that PLC/PRF/5 hepatoma cells show a resistance to Fas-mediated apoptosis that is due to a functional absence of FADD.

Materials and Methods

Cell Culture. Two human hepatoma cell lines, HepG2 and PLC/PRF/5 cells, were kindly supplied by Dr. Yoshihide Tsujimoto, Osaka University Medical School, Osaka, Japan. Cells were maintained in RPMI-1640 medium (Gibco BRL, Life Technologies, Inc., Rockville, MD) and supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) in a humidified atmosphere of 5% CO_2 and 95% air.

Assay of Cell Viability. Apoptotic cell death is accompanied by condensation and/or fragmentation of chromosomal DNA (1, 20). In the present study, nuclei of treated cells were stained with Hoechst 33342/propidium iodide (PI) using procedures described previously (21–23). Nuclei without condensed and/or fragmented nuclei were recognized as normal. Cell viability was quantitated as the percentage of normal nuclei in total (about 5000) nuclei. Hoechst 33342 and PI were purchased from Molecular Probes, Inc. (Eugene, OR). After stimulation with antihuman Fas antibody (Fas Ab; CH-11 clone, MBL, Nagoya, Japan and Ref. 24), cells were collected and stained with Hoechst 33342/PI, and then observed by fluorescence microscopy.

Cell viability was also measured with an MTT assay that was performed as previously described (22, 23).

Preparation of Antihuman ILP Antibody. The pGEX5X-3 (25) was digested with BamHI, blunted with the Klenow fragment, and then self-ligated. The resulting plasmid was named pGEX5X-3′. The *myc*-epitope tagged ILP cDNA (pcDNAs-mycILP) was the kind donation of Dr. C. B. Thompson, The University of Chicago, Chicago, Illinois (25). A mycILP fragment was isolated by EcoRI digestion and subcloned into pGEX5X-3′. The GST-mycILP fusion protein was expressed in *E. coli* and purified on glutathione Sepharose 4B columns. An antigen emulsion was made from the purified protein (100 μg) and complete Freund adjuvant (for primary immunization) or incomplete Freund adjuvant (for booster immunization) using an ultrasonicator. Female 8-week-old Balb/c mice were immunized subcutaneously. Booster immunizations were given 2 and 5 weeks later, and blood was collected 1 week after the final booster immunization.

Protein Extraction. Proteins were prepared for immunoblotting and immunoprecipitation analysis by lysing cells with 1% NP-40 containing PBS for 30 min. All procedures were carried out at 4°C. Lysates were centrifuged at 15000 rpm for 15 min. Protein concentrations were determined by a method described previously (26, 27), using the BCA protein assay kit (Pierce, IL) with bovine serum albumin as the standard.

Immunoblotting Analysis. Sample proteins separated by SDS-PAGE were transferred onto nitrocellulose filters with a semidry blotting system. The blotted filters were blocked with PBS containing 5% (w/v) skim milk (Snow Brand, Sapporo, Japan) at room temperature for 1 hr, washed with a mixture of PBS and 0.05% Tween 20 (Tween-PBS, Sigma-Aldrich Japan, Tokyo, Japan), and then incubated overnight at room temperature with each antibody diluted with PBS. After washing with Tween-PBS, the filters were incubated with a 1000-fold diluted biotinylated anti-mouse IgG antibody (Bio Source, Camarillo, CA), washed with Tween-PBS, and then incubated with avidin-horseradish peroxidase (Vector Lab, Inc., Burlingame, CA) at room temperature for 1 hr. The filters were washed with Tween-PBS and then developed with an ECL substrate and detection system. Antibodies specific for Fas, Bcl-2, Bcl-xL, Bcl-xs, Bax, and Bak were purchased from CalBiochem (San Diego, CA); antibody specific for caspase 3 was purchased from Signal Transduction Lab., (Lexington, KY).

Far-Western Blotting Analysis Using FDD. Proteins extracted from HepG2 or PLC/PRF/5 cells were separated on 10% polyacrylamide gels and transferred onto a nitrocellulose filter using a semidry blotting system. Proteins were renatured with PBS containing 3% NP-40, and filters were blocked with skim milk and incubated with 1 μg/ml of biotinylated recombinant human Fas-death domain (FDD) purchased from Wako (Osaka, Japan). Detection of bound biotin-FDD was performed with avidin-horseradish peroxidase and ECL as described above.

Preparation of FADD Protein. To investigate effects of FADD in Fas-mediated cell death of two hepatoma cell lines, FADD protein was collected from HepG2 cells.

Proteins were extracted with 1% NP40 in PBS from HepG2 cells, and then FADD was collected with anti-FADD antibody-conjugated protein A-Sepharose column. FADD was eluted from the column with a sodium chloride gradient and desalted. Monoclonal antibody for FADD was purchased from Transduction Lab.

Microinjection Analysis. Microinjection analysis was performed for the investigation of FDD- and FADD-effects in Fas-mediated cell death in two hepatoma cell lines. FDD and FADD were diluted in PBS at 1 mg/ml, and microinjection was performed with a Transjector 5246 (eppendorf, Hamburg, Germany). Microinjection was performed at 100 hPa for 3 s. In the present study, no abnormalities were observed during the first week after the cells were microinjected with PBS.

Results

Fas Ab–Induced Apoptotic Cell Death in HepG2 Cells, but not in PLC/PRF/5 Cells. The effect of the agonistic antihuman Fas antibody (Fas Ab: CH-11 clone and Ref. 24) in two human hepatoma cell lines was examined. Fas Ab treatment in the absence of actinomycin D, a *de novo* protein synthesis inhibitor (transcription inhibitor), did not decrease cell viability in either hepatoma cell line (data not shown). However, when cells were treated with various doses of Fas Ab for 24 hr in the presence of actinomycin D, dose-dependent cytolytic activity was observed only in HepG2 cells, but not in PLC/PRF/5 cells (Fig. 1A). To further examine the effects of the Fas Ab in both hepatoma cell lines, Hoechst 33342/PI staining analysis was performed. As shown in Fig. 1(B), Fas Ab induced a decrease in the numbers of intact nuclei in HepG2 cells, but not in PLC/PRF/5 cells. Both observations indicate that Fas-mediated apoptosis is initiated in only HepG2 cells, but not in PLC/PRF/5 cells.

Caspase 3 and 8 in Both Hepatoma Cell Lines. Although there were no obvious differences in Fas expression in the two hepatoma cell lines (Fig. 2A), a significant difference was observed in Fas-sensitivity. To further investigate this difference, the expression and activation of caspase 3 and 8 was examined. As shown in Fig. 2(B,C), expression of caspase 3 and 8 was observed in both hepatoma cell lines, but these were activated in only HepG2 cells after Fas Ab treatment. These results suggest that PLC/PRF/5 cells either lack a necessary factor(s) that participates in the signaling pathway of caspase 3 and 8 activation, or overexpress the death suppressor.

Comparison of Death Suppressor Expression. To examine the possibility that the resistance to Fas in the PLC/PRF/5 cells was due to an overexpression of the death suppressor, immunoblotting analysis was performed. As shown in Table I of the death suppressors and their related proteins that were tested (death suppressor: Bcl-2, Bcl-xL, and ILP; and Bcl-2 related protein: Bcl-xs, Bax and Bak), there were no apparent differences in expression between HepG2 and PLC/PRF/5 cells.

Functional Absence of FADD in PLC/PRF/5 Cells. Fas contains multiple domains, and the cytoplasmic death domain is the most important for the transduction of apoptotic death signaling (2, 12, 13). We therefore examined Fas-death domain (FDD)-binding proteins in HepG2 and PLC/PRF/5 cells. Far-Western blotting analysis revealed two FDD-binding proteins (46 kDa and 27 kDa) in HepG2 cells (Fig. 3A). In contrast, PLC/PRF/5 cells showed only a 46-kDa FDD-binding protein (Fig. 3A). On the basis of molecular weight comparison (28) and immunoblotting analysis, it appears that the 46-kDa protein is Fas, and the 27-kDa protein is FADD (Figs. 2A and 3B).

Regulation of Apoptosis by FADD in Hepatoma Cells. Our present findings suggest that the resistance to Fas-mediated apoptosis in PLC/PRF/5 cell is due to a functional absence of FADD. Therefore, an effect of FDD (human recombinant Fas-death domain) or FADD in Fas-mediated apoptosis of hepatoma cells was examined to evaluate this possibility. Results from the microinjection of FDD or FADD into each hepatoma cell line showed that HepG2 cells are resistant to Fas-mediated apoptosis, even in the presence of actinomycin D by FDD, and Fas sensitivity was acquired by FADD in PLC/PRF/5 cells (Fig. 4). These results strongly suggest that the resistance to Fas-mediated

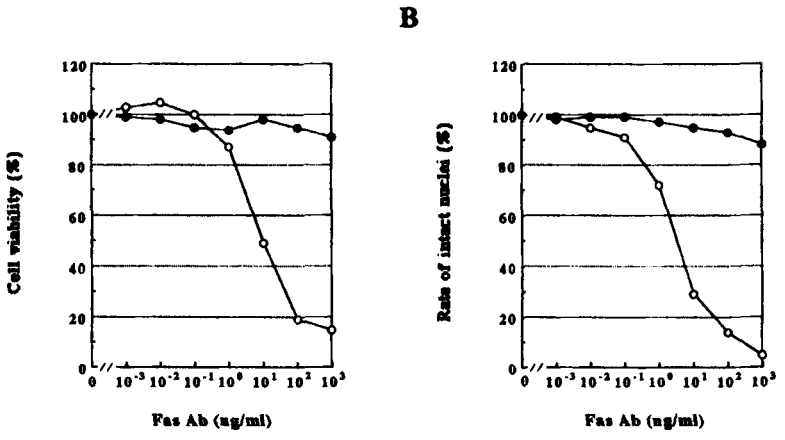
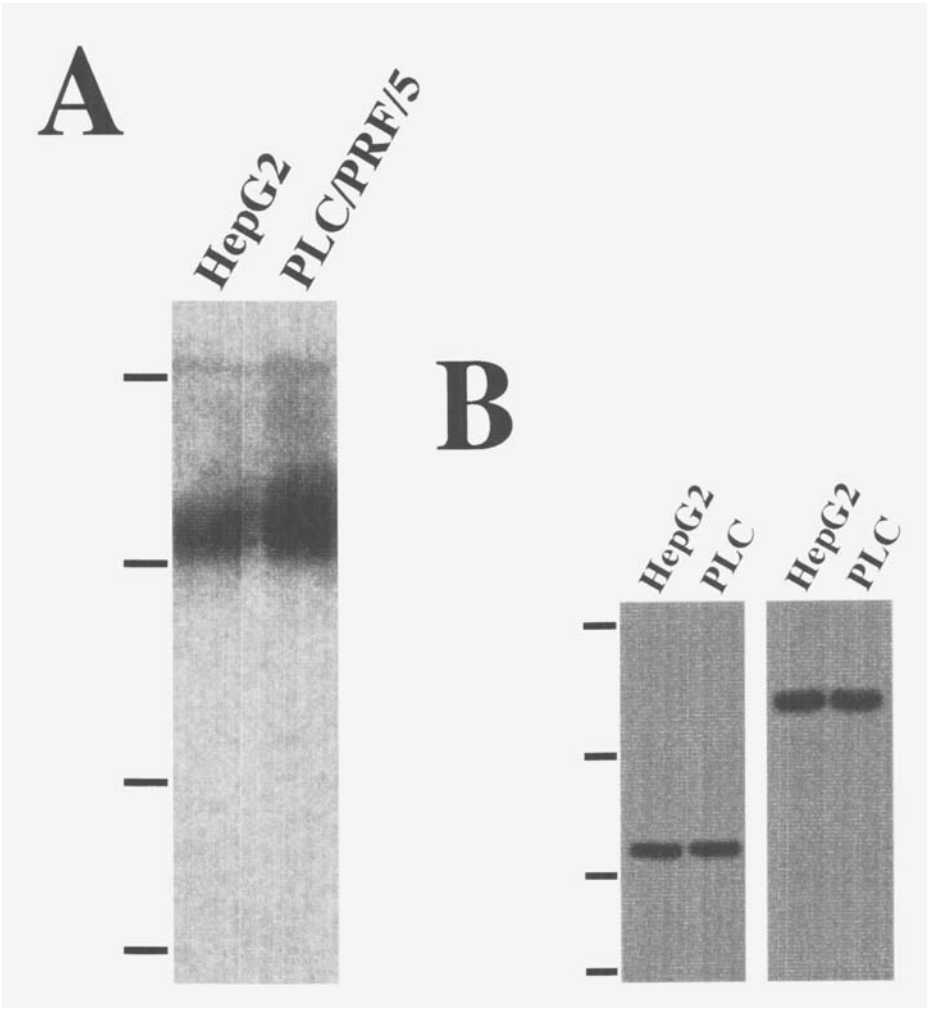


Figure 1. Fas-mediated apoptosis in human hepatoma cell lines. HepG2 (open circle) or PLC/PRF/5 (closed circle) cells were treated with various concentrations of agonistic Fas antibody (CH-11 clone) for 24 hr in the presence of actinomycin D (0.5 μg/ml). After treatment, (A) cell viability and (B) percentage of intact nuclei were measured with the (A) MTT assay or the (B) Hoechst 33342/PI staining procedure.



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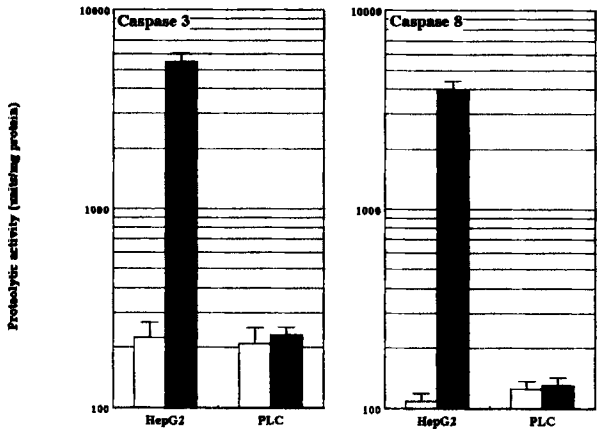

Figure 2. Death inducers in hepatoma cell lines. (A) Expression of Fas. Proteins were extracted from HepG2 or PLC/PRF/5 cells, and immunoblotting was performed. Bars on the left side of the photograph show the protein marker positions (66, 45, 30 and 20 kDa; top to bottom). (B) Expression of caspase 3 and 8. Immunoblotting with antibodies to caspase 3 (left) and 8 (right) was performed. Bars on the left side of the photograph show the protein marker positions (66, 45, 30 and 20 kDa; top to bottom). (C) Activation of caspase 3 and 8. Cells were treated with (closed) or without (open) Fas Ab (1 μg/ml) for 12 hr in the presence of actinomycin D (0.5 μg/ml). Enzymatic activities of caspase 3 (left) and 8 (right) were measured with DEVD-MCA (substrate for caspase 3 and Refs. 21 and 42) or IETD-MCA (substrate for caspase 8 and Refs. 23 and 43).

apoptosis in PLC/PRF/5 cells is due to a functional absence of FADD.

Discussion

The death receptor Fas induces apoptotic death signaling upon stimulation by the Fas ligand (2). Recently, a dominant role of Fas in various disease states, especially in hepatitis, has been reported (2, 5). Hepatitis virus, including HBV, initiates viral antigen expression on the surface of infected hepatocytes. CTL recognize this cell surface antigen, and the Fas ligand/Fas system is triggered by the contact of CTL and hepatitis virus–infected hepatocytes to remove infected cells (10, 11). However, in rare instances, some hepatitis virus–infected hepatocytes avoid this death induction machinery and transform to hepatoma by mechanisms that are largely unknown. The PLC/PRF/5 cell line is known to be an HBV-infected and antigen-expressing hu-

Table I. Immunoblotting Analysis of Death Suppressors and *Bcl*-2-Related Proteins

Family	Antibody specificity	HepG2	PLC/PRF/5	Function
Bcl-2				
	Bcl-2	–	–	Suppressor
	Bcl-xL	–	–	Suppressor
	Bcl-xs	–	–	Accelerator
	Bax	+	+	Accelerator
	Bak	+	+	Accelerator
IAP				
	ILP	+	+	Suppressor

Note. – = not detected; + = detected.

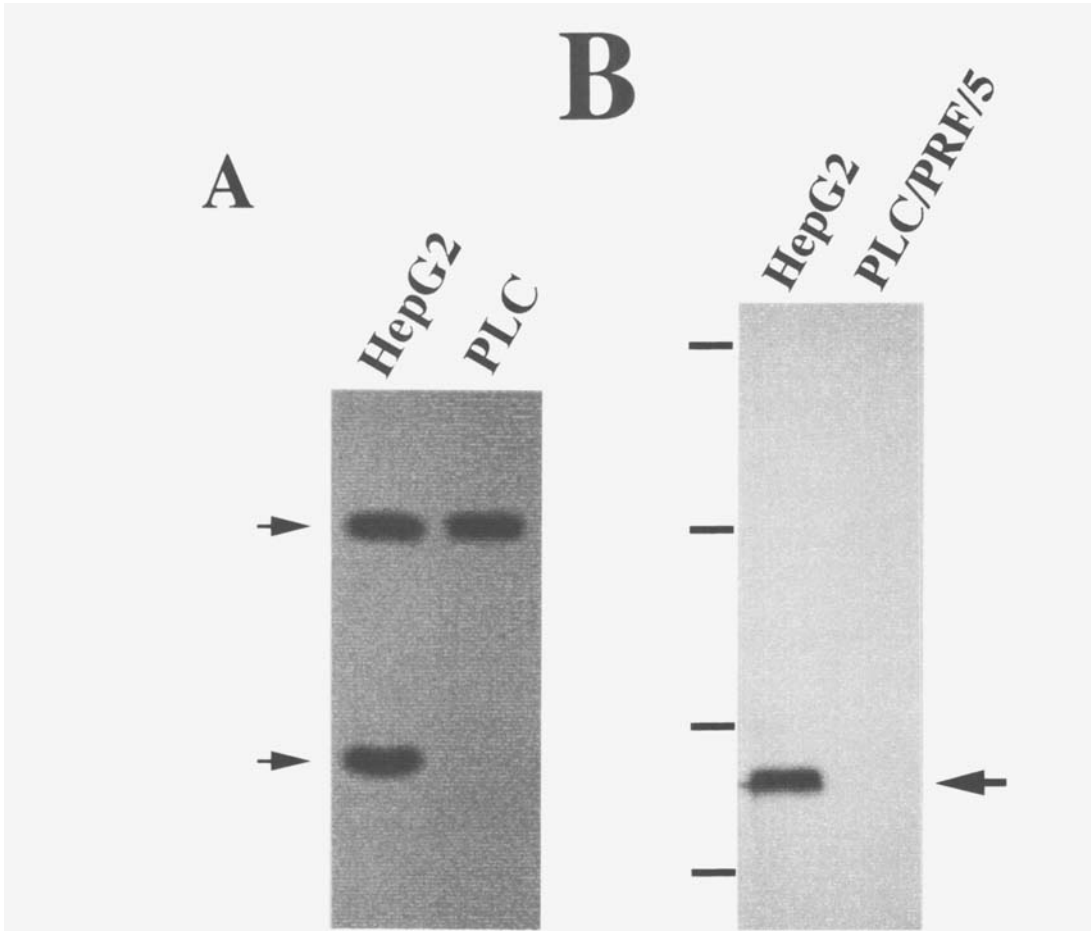

Figure 3. (A) Far-Western blotting analysis using FDD. Proteins extracted from HepG2 (HepG2) or PLC/PRF/5 (PLC) cells were separated on 10% SDS-PAGE gels, renatured, and then incubated with biotinylated FDD. Arrowheads show the position of FDD-binding proteins (upper is 46-kDa, and lower is 27-kDa). (B) Absence of FADD in PLC/PRF/5 cells. Proteins from HepG2 or PLC/PRF/5 cells were separated on 10% SDS-PAGE gels and reacted with antihuman FADD. The arrow shows the position of FADD, and bars on the left side show the protein marker positions (66, 45, 30 and 20 kDa; top to bottom).

man hepatoma cell line (17–19), and therefore, useful to achieve the molecular mechanism of transformation in HBV-infected hepatocytes.

Fas-mediated apoptosis was examined using an agonistic antihuman Fas antibody (Fas Ab: CH-11 clone and Ref. 24) in two human hepatoma cell lines, HepG2 and PLC/PRF/5 cells. HepG2 cell viability decreased by the Fas Ab treatment in the presence of actinomycin D with a parallel reduction in the percentage of intact nuclei. Fas Ab induced nuclear condensation and/or fragmentation in a typical apoptotic fashion, and in a dose-dependent manner, consistent with results reported by Hasegawa *et al.* (21). In contrast, PLC/PRF/5 cells did not show any decrease in cell viability or percentage of intact nuclei. These results raise the possibility that a comparison between HepG2 and PLC/PRF/5 cells may provide a model system to achieve the molecular mechanisms of the transformation of HBV-infected hepatocytes.

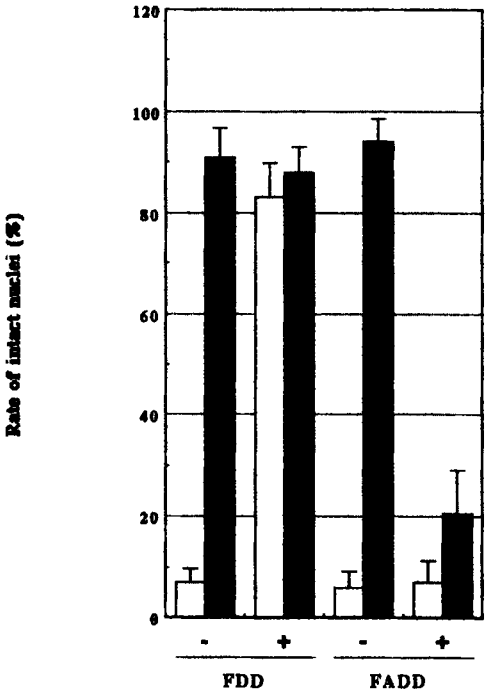

Figure 4. Regulation of Fas-mediated apoptosis by FDD and FADD. FDD or FADD was loaded into HepG2 (open) or PLC/PRF/5 (closed) cells by microinjection. After microinjection, cells were treated with Fas Ab at 1 μg/ml for 24 hr, and then the percentage of intact nuclei was calculated with the Hoechst 33342/PI staining procedure.

Immunoblotting analysis was performed as an initial approach. We examined the expression of nine death-associated factors: Fas, Bcl-2, Bcl-xL, Bcl-xs, Bax, Bak, caspase 3, caspase 8 and ILP. Immunoblotting analysis did not reveal any apparent differences in expression of these antigens between HepG2 and PLC/PRF/5 cells. However, several conclusions regarding the molecular mechanism of the transformation of HBV-infected hepatocyte can be drawn from these observations: 1) HBV may not affect Fas expression, including Fas mRNA transcription and translation, since there is no difference in Fas expression between HepG2 and PLC/PRF/5 cells; 2) The death suppressors Bcl-2 and Bcl-xL (29–31) were not expressed in these hepatoma cell lines so the Bcl-2 family, especially suppressors, is not involved in the resistance of PLC/PRF/5 cells; 3) Some death accelerators (31–33) (Bax and Bak, but not Bcl-xs) were expressed in both hepatoma cell lines, but these are also not involved with the resistance; and 4) PLC/PRF/5 cells carry the endogenous suppressor ILP for caspase 3 (34). However, these factors are most likely not involved with the resistance of PLC/PRF/5 cells, since HepG2 cells, which demonstrate Fas-mediated apoptosis, showed similar antigen expression.

In addition, activation of the caspase 3 and 8 death mediators was also examined. Following death induction in HepG2 cells, activation of both caspase 3 and 8 was observed. Whereas expression of caspase 3 and 8 was detected in PLC/PRF/5 cells, neither death mediator was activated by the Fas Ab treatment. These results raised the possibility that PLC/PRF/5 cells may lack the factor(s) involved with the caspase activation transduced from Fas. Fas contains some interesting domains including an extracellular domain for ligand binding (12), an inhibitor domain for the binding of FAP-1 (35), and the most important region for the transduction of apoptotic death signaling, the "death domain" (12, 13). Recently, FADD was identified as a Fas-death domain (FDD)–binding protein (14). FADD binding to the FDD supports the apoptotic death signaling by transduction of the death stimulation to a downstream death-associated factor, such as caspase 8 (15, 16). Thus, FDD-associated factors exist in the cytoplasm. The above tested factors, except for caspase 8, act in key roles at the down-stream portion of the pathway, as compared with FDD-associated factors like FADD. Far-Western blotting analysis revealed two FDD-associated proteins in HepG2 cells (46-kDa and 27-kDa). Interestingly, the 27-kDa FDD-associated protein did not exist in PLC/PRF/5 cell extracts, suggesting the possible involvement of this protein in the resistance to Fas-mediated apoptosis in PLC/PRF/5 cells. On the basis of molecular weight comparison (28) and ability to bind to FDD, the possibility was raised that the 46-kDa protein was Fas and the 27-kDa protein was FADD. Immunoblotting analysis additionally confirmed the identities of these proteins and indicated the functional absence of FADD in PLC/PRF/5 cells. In addition, our present results strongly suggest that the resistance to Fas-mediated apoptosis in PLC/PRF/5 cells is due to the functional absence of FADD. Although PLC/PRF/5 cells carry caspase 8, they cannot mediate the apoptotic death signaling in the absence of FADD.

We suggested that the resistance to Fas-mediated apoptosis in PLC/PRF/5 cell is due to a functional absence of FADD. This observation may give some insight into the molecular mechanism leading to the transformation of HBV-infected hepatocytes. HepG2 cells show Fas-mediated cell death only in the presence of actinomycin D. However, even those cells showed resistance in the presence of actinomycin D after FDD injection. Using Far-Western blotting, we demonstrated that FDD is bound to Fas, possibly to the cytoplasmic "death domain," and FADD. One explanation is that this resistance is due to the binding of FDD to the Fas-death domain and FADD. In other words, FDD acted as a pseudo-target for FADD and Fas-death domain in HepG2 cells. Thus, it appears that preventing the binding between the Fas-death domain and FADD results in resistance to Fas-mediated apoptosis. On the other hand, PLC/PRF/5 cells showed the resistance to Fas-mediated apoptosis, but Fas sensitivity was acquired by FADD injection. These results strongly indicated that PLC/PRF/5 cells lack FADD following HBV-induced carcinogenesis.

In the present study, we attempted to elucidate the molecular mechanism of the transformation of HBV-infected hepatocytes to hepatoma, using the human HBV-infected hepatoma PLC/PRF/5 cell line. We compared the Fas-sensitive hepatoma HepG2 cells and Fas-insensitive hepatoma PLC/PRF/5 cells, and demonstrated a functional ab-

sence of FADD in the PLC/PRF/5 cells. On the basis of our results, we hypothesize that the molecular mechanism of the HBV-infected hepatocyte transformation may proceed as shown schematically (Fig. 5). When HBV infects a hepatocyte, expression of HBV-cell surface antigen is induced. CTLs recognize this antigen and bind to the HBV-infected hepatocyte. Upon contact between the HBV-infected hepatocyte and the CTL, the Fas ligand binds to Fas to initiate the apoptotic death signaling events for removal of the HBV-infected hepatocyte. This hepatocyte death results in hepatitis. In contrast, HBV infection may also block FADD by an unknown system resulting in a functional absence of FADD. These HBV-infected hepatocytes avoid Fas-mediated apoptosis, and ultimately transform to hepatoma. Recently we reported that PKC-ε was newly expressed during the malignancy of mammary tumor (36). PKC-ε may initiate the transformation of HBV-infected hepatocyte after the avoidance of Fas-mediated apoptosis.

This approach is just one of numerous molecular mechanisms for transformation of HBV-infected hepatocytes. Recently, various death suppressors have been identified from viral genes, such as cowpox virus *crmA* and baculovirus *p35* that directly suppress the interleukin-1β converting enzyme (ICE: caspase 1 37–40), and FLIP that suppresses the binding of Fas-death domain and caspase 8 (41). Thus, some viral proteins can directly suppress Fas-mediated apoptosis. HBV appears to suppress Fas-mediated apoptosis indirectly, namely death suppression by the induction of FADD dysfunction. Further studies are needed to elucidate the molecular pathways that support this role of FADD in HBV infection.

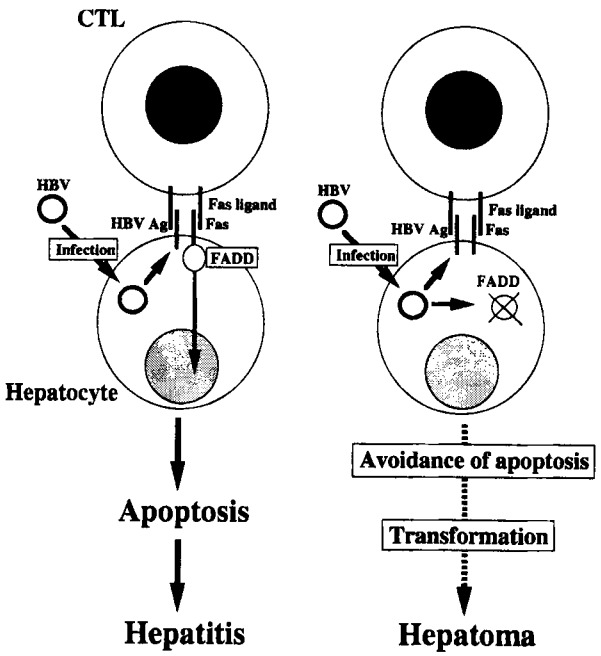

Figure 5. Schematic model of the molecular mechanism of HBV-infected hepatocyte death and transformation. HBV infects hepatocytes and induces the surface expression of HBV antigen for recognition by CTLs. The Fas ligand expressed on CTL cell surfaces binds in a reciprocal fashion to Fas receptor expressed on the infected hepatocytes. Appropriate signal transduction occurs, and hepatitis is induced as a result of apoptosis (left model). In contrast, HBV blocks functional expression of FADD in some HBV-infected hepatocytes. The cells then transform to hepatoma as a result of the avoidance of Fas-mediated apoptosis. Abbreviations are: CTL: cytotoxic T lymphocyte; HBV: hepatitis B virus; HBV Ag: HBV antigen.

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1. Wyllie AH, Kerr JFR, Currie AR. Cell death. The significance apoptosis. Int Rev Cytol **68:**251–306, 1980.
2. Nagata S, Golstein P. The Fas death factor. Science **267:**1449–1456, 1995.
3. Suzuki A, Enari M, Eguchi Y, Matsuzawa A, Nagata S, Tsujimoto Y, Iguchi T. Involvement of Fas in regression of vaginal epithelia after ovariectomy and during an estrous cycle. EMBO J **15:**211–215, 1996.
4. Suzuki A, Matsuzawa A, Iguchi T. Downregulation of *Bcl-2* is the first step on Fas-mediated apoptosis of male reproductive tracts. Oncogene **13:**31–37, 1996.
5. Suzuki A, Enari M, Iguchi T. Effect of neonatal exposure to DES on Fas and *Bcl-2* expression in the adult mouse vagina: An approach to the DES syndrome. Reprod Toxicol **10:**465–470, 1996.
6. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. Lethal effect of the anti-Fas antibody in mice. Nature **364:**806–809, 1993.
7. Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. Nat Med **3:**409–413, 1997.
8. Rodriguez I, Matsuura K, Ody C, Nagata S, Vassalli P. Systemic injection of a tripeptide inhibits the intracellular activation of CPP32-like proteases *in vivo* and fully protects mice against Fas-mediated fulminant liver destruction and death. J Exp Med **184:**2067–2072, 1996.
9. Suzuki A. The dominant role of CPP32 subfamily in Fas-mediated hepatitis. Proc Soc Exp Biol Med **217:**450–454, 1998.
10. Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, Fusamoto H, Kamada T. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology **19:**354–1359, 1994.
11. Berke G. The CTL's kiss of death. Cell **81:**9–12, 1995.
12. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell **66:**233–243, 1991.
13. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature **356:**314–317, 1992.
14. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain–containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell **81:**505–512, 1995.
15. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell **85:**817–827, 1996.
16. Boldin MP, Goncharov TM, Golstev YV, Wallach D. Involvement of MACH, a novel MORT-1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell **85:**803–815, 1996.
17. Gousevv AI, Engelhardt NV, Masseyeff R, Camain R, Basteris B.

Immunofluorescent study of α-foetoprotein (α-fp) in liver and liver tumors. II. Localization of α-fp in the tissues of patients with primary liver cancer. Int J Cancer **7:**207–217, 1971.
18. Hajnicka V, Fuchsberger N, Stancek D, Volna A, Cervenakova L. Synthesis of 23K acute-phase protein by HBV genome carrying PLC/PRF/5 human hepatoma cells. Acta Virol **36:**581–584, 1992.
19. Tugizov SM, Savchenkova IP, Grabovskaya IL, Makarova NE, Eraizer TL, Revazova EP, Kushch AA. Changes in expression of surface and core antigens of hepatitis B virus in different mutant clones of hepatoma PLC-PRF-5 cells. Virus Res **30:**189–203, 1993.
20. Fesus L. Biochemical events in naturally occurring forms of cell death. FEBS Lett **328:**1–5, 1993.
21. Hasegawa J, Kamada S, Kamiike W, Shimizu S, Imazu T, Matsuda H, Tsujimoto Y. Involvement of CPP32/Yama(-like) proteases in Fas-mediated apoptosis. Cancer Res **56:**1713–1718, 1996.
22. Suzuki A, Iwasaki M, Kato M, Wagai N. Sequential operation of ceramide synthesis and ICE cascade in CPT-11-initiated apoptotic death signaling. Exp Cell Res **233:**41–47, 1997.
23. Suzuki A, Iwasaki M, Wagai N. Involvement of cytoplasmic serine-proteinase and CPP32 subfamily in the molecular machinery of caspase 3 activation during Fas-mediated apoptosis. Exp Cell Res **233:**48–55, 1997.
24. Yonehara S, Ishii A, Yonehara M. A cell killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med **169:**1747–1756, 1989.
25. Duckett CS, Nava VE, Gedrich RW, Clem RJ, Dongen JLV, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB. A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors. EMBO J **15:**2685–2694, 1996.
26. Hill HD, Sraka J. Protein determination using bicinchoninic acid in the presence of sulfhydryl regents. Anal Biochem **170:**203–208, 1988.
27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem **170:**203–208, 1985.
28. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J **14:**5579–5588, 1995.
29. Tsujimoto Y, Yunis J, Nowell PC, Croce CM. Cloning of the chromosomal breakpoint of neoplastic B cells with the t(14; 18) chromosome translocation. Science **226:**1097–1099, 1984.
30. Tsujimoto Y. Stress-resistance conferred by high level of *Bcl*-2 α-protein in human B lymphoblastoid cell. Oncogene **4:**1331–1336, 1989.
31. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. *Bcl*-x, a *Bcl*-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell **74:**597–608, 1993.
32. Oltvai ZN, Milliman CL, Korsmeyer SJ. *Bcl*-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell **74:**609– 619, 1993.
33. Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, Guild BC. Induction of apoptosis by the *Bcl*-2 homologue Bak. Nature **374:**733–736, 1996.
34. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell death proteases. Nature **388:**300–304, 1997.
35. Sato T, Irie S, Ktada S, Reed JC. FAP-1: A protein tyrosine phosphatase that associates with Fas. Science **268:**411–415, 1995.
36. Suzuki A, Goto Y, Iguchi T. Progression of PDMT is accompanied by lack of Fas and intense expression of *Bcl*-2 and *PKC*-ε. Carcinogenesis **18:**883–887, 1997.
37. Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ. Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell **69:**597–604, 1992.
38. Xue D, Horvitz HR. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature **377:**248–251, 1995.
39. Tewari M, Dixit VM. Fas- and TNF-induced apoptosis is inhibited by the Poxvirus crmA gene product. J Biol Chem **270:**3255–3260, 1995.
40. Enari M, Hug H, Nagata S. Involvement of an ICE-like proteinase in Fas- mediated apoptosis. Nature **375:**78–81, 1995.
41. Irmler M, Thome M, Hahne M, Schneider P, Hofman K, Steiner V, Bodmer J-L, Schroter M, Burns K, Mattman C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature **388:**190–195, 1997.
42. Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M. Resistance to Fas- mediated apoptosis: Activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. Oncogene **17:**931–940, 1998.
43. Thornbery NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstorm PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. A combinatorial approach defined specificities of members of the caspase family and granzyme B: Functional relationships established for key mediators of apoptosis. J Biol Chem **272:**17907–17911, 1997.