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Cellular FLICE/Caspase-8–Inhibitory Protein as a Principal Regulator of Cell Death and Survival in Human Hepatocellular Carcinoma

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SUMMARY: Human hepatocellular carcinomas (HCCs) show resistance to apoptosis mediated by several death receptors. Because cellular FLICE/caspase-8–inhibitory protein (cFLIP) is a recently identified intracellular inhibitor of caspase-8 activation that potently inhibits death signaling mediated by all known death receptors, including Fas, TNF-receptor (TNF-R), and TNF-related apoptosis-inducing ligand receptors (TRAIL-Rs), we investigated the expression and function of cFLIP in human HCCs. We found that cFLIP is constitutively expressed in all human HCC cell lines and is expressed more in human HCC tissues than in nontumor liver tissues. Metabolic inhibitors, actinomycin D (ActD) or cycloheximide (CHX), dramatically rendered HCC cells sensitive to Fas-mediated apoptosis. Neither caspase-8 nor caspase-3 was activated by agonistic anti-Fas antibody alone, but both caspases were activated by Fas stimulation in the presence of ActD or CHX, indicating the importance of caspase-8 inhibitors that are sensitive to metabolic inhibitors. Actually, cFLIP expression was decreased in ActD or CHX treatment. cFLIP down-regulation induced by cFLIP antisense oligodeoxynucleotides sensitized HLE cells to Fas, TNF-R, and TRAIL-R–mediated apoptosis. Furthermore, cFLIP over-expression activated nuclear factor (NF)-κB and cFLIP down-regulation attenuated NF-κB activation induced by TNF-α or TRAIL. Pretreatment with pan-caspase-inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD-fmk), restored NF-κB activity attenuated by cFLIP down-regulation. cFLIP expression was increased by TNF-α, TRAIL, or vascular endothelial growth factor but decreased by wortmannin, indicating that cFLIP expression is regulated by both the NF-κB and phosphatidylinostiol-3 kinase (PI-3)/Akt pathways. These results suggest that cFLIP plays an important role in cell survival not simply by inhibiting death-receptor–mediated apoptosis but also by regulating NF-κB activation in human HCCs. (*Lab Invest 2003, 83:1033–1043*).

Apoptosis, or programmed cell death, is a key mechanism regulating various physiologic events, including the elimination of unwanted cells, defense against agents of infectious disease and cancers, as well as the maintenance of tissue homeostasis (Nagata, 1997; Vaux et al, 1994). Apoptotic cell death can be triggered by the engagement of any one of several cell surface death receptors, all of which express extracellular cysteine-rich pseudorepeats, contain homologous intracellular C–terminal death domains, and are members of the TNF-receptor (TNF-R) family. These receptors include Fas, TNF-receptor 1 (TNF-R1), TNF-related apoptosis-induced ligand (TRAIL)-receptor 1 (R1), TRAIL-R2, DR3, and DR6 (Bazzani and Beutler, 1996; Gura, 1997; Inoue et al, 2000; Locksley et al, 2001). Stimulation of Fas and TRAIL-R1 or -R2 results in clustering of the receptor, which in turn leads to recruitment of the adaptor molecule and Fas-associated death domain (FADD) and causes the receptor proximal caspase-8 to become activated upon recruitment to the death-inducing signaling complex (DISC). Active caspase-8 dissociates from DISC to start the activation of a caspase cascade, resulting in the execution phase of apoptosis (Chinnaiyan et al, 1996; Kischkel et al, 1995; Medema et al, 1997; Muzio et al, 1996; Thornberry and Lazebnik, 1998).

Recently, it has been shown that FLICE/caspase-8 inhibitory protein (FLIP), a part of DISC, is crucial for modulation of the cell death signal, inhibiting procaspase-8 processing at DISC. At first, viral proteins that contain death-effector domains were discovered in herpesvirus saimiri, human herpesvirus 8, Kaposi's sarcoma–associated herpesvirus, and molluscum contagiosum virus by screening public databases. These were named "viral FLIP" (vFLIP) (Thome et al, 1997). A human cellular homolog of vFLIP was later found and termed "cellular FLIP," (cFLIP; also called FLAME-1, I-FLICE, Casper, or CASH) (Goltsev et al, 1997; Hu et al, 1997; Irmler et al, 1997; Shu et al, 1997; Srinivasula et al, 1997). cFLIP expression af-

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fords apoptotic tolerance from known death receptors, Fas, TRAIL-R, TNFR1, and DR3 to apoptosis-sensitive cells (Irmler et al, 1997).

cFLIP is predominantly expressed in the heart, skeletal muscle, and peripheral blood leukocytes (Irmler et al, 1997). Hence, cFLIP is thought to be involved in the regulation of the immune system. A recent study has demonstrated that the resistance of dendritic cells against Fas-mediated apoptosis is associated with FLIP expression (Willems et al, 2000); it also showed that B cell receptor signaling up-regulates FLIP and suppresses the Fas- and TRAIL-receptor apoptosis pathway, which could be important for tolerance and selection of antigen-specific B cells (Wang et al, 2000). These results suggest that cFLIP plays an important physiologic role in rescuing cells from death-receptor–mediated apoptosis.

Dysregulation of apoptosis signaling is often associated with disease formation, such as autoimmune diseases (Krammer, 1999) and cancer (Thompson, 1995). In hepatocarcinogenesis, failure of apoptosis is considered to be important for the survival of hepatocytes that are prone to undergo genetic damage and cellular transformation by an increased proliferative rate due to hepatocyte regeneration (Schulte-Hermann et al, 1997; Thorgeirsson et al, 1998). In fact, most hepatocellular carcinoma (HCC) cells show strong resistance to various stimuli that otherwise induce apoptosis. Several cellular antiapoptotic mechanisms such as reduced expression of Fas (Strand et al, 1996; Thorgeirsson et al, 1998) or caspases (Fujikawa et al, 2000), as well as expression of antiapoptotic proteins, the Bcl-2 family (Soini et al, 1996; Takehara et al, 2001), and inhibitor of apoptosis families (Ito et al, 2000; Shiraki et al, 2002), or receptor-mediated survival signals (Sugimoto et al, 1999; Suzuki et al, 2000) are known to contribute to resistance against immunologic cytotoxicity in human HCC cells. Expression of cFLIP is reported to be highly correlated with malignant potential in colonic adenocarcinomas (Ryu et al, 2001) and melanomas (Bullani et al, 2001). All of this evidence suggests that cFLIP might therefore account for the development of malignant diseases by regulating the apoptosis signaling pathway. However, the expression and biological function of cFLIP in tumor cells has not been well elucidated.

On the other hand, the transcriptional factor nuclear factor (NF)-κB also plays an important role in tumor cell survival. NF-κB activation is induced by known signaling pathways, including Fas, TRAIL-R, or TNF-R1 (Ponton et al, 1996; Wajant et al, 2000). NF-κB activation induces specific gene expression to regulate programmed cell death tightly and is associated with inhibition of apoptosis (Barkett and Gilmore, 1999; Beg et al, 1995; Schmid and Adler, 2000). Attention should be called to the fact that TNF receptor families have a characteristic ability to induce two opposite effects, either apoptosis or antiapoptosis. The role of cFLIP in switching these death-receptor–mediated pathways has not been clarified either. Therefore, to gain insight into the biological role of cFLIP in malignant cells, we investigated the expression of cFLIP and its function in TNF family–induced apoptosis and NF-κB activation in human HCCs. Furthermore, we studied the regulation of cFLIP expression by several mediators.

Results

Induction of Apoptosis by Anti-Fas Antibody in HCC Cells

At first we investigated cytotoxicity of an anti-Fas agonistic antibody in HCC cells. As shown in Figure 1, anti-Fas agonistic antibody alone could not induce apoptosis in human HCC cells. Next, the effects of an RNA synthesis inhibitor, like actinomycin D (ActD), or protein synthesis inhibitor, like cycloheximide (CHX), on anti-Fas agonistic antibody-induced apoptosis was examined. We incubated human HCC cells (SK-Hep1, HLE, or HepG2) with 0.5 μg/ml ActD or 2.0 μg/ml CHX, doses that are clinically relevant in patients, in combination with 100 ng/ml anti-Fas agonistic antibody for 24 hours at 37° C; cell viability was also analyzed (Fig. 1). The cytotoxicity of the inhibitors alone, such as ActD, CHX, and anti-Fas agonistic antibody, was low in three HCC cell lines; however, in the presence of a subtoxic level (0.5 μg/ml ActD or 2.0 μg/ml CHX) of metabolic inhibitors, the effect of the combination of anti-Fas agonistic antibody and these metabolic inhibitors was synergistic compared with

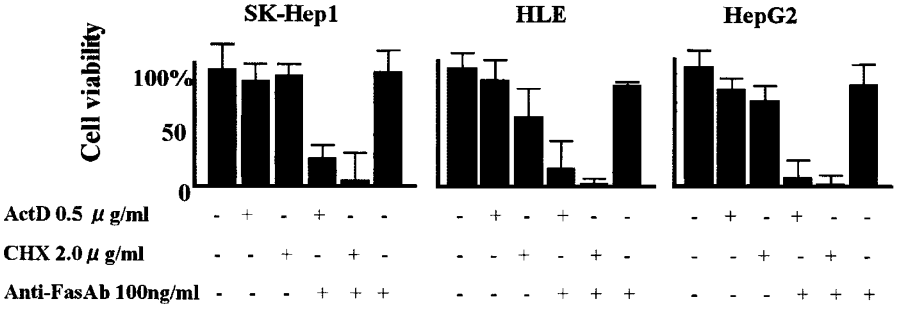

Figure 1.

The cell viability exchange of HCC cells (SK-Hep1, HLE, and HepG2) by incubation with 0.5 μg/ml actinomycin D, 2.0 μg/ml cycloheximide or 100 ng/ml anti-Fas agonistic antibody. Cell viability was assessed by the MTT assay. Data shown are the mean ± SD of eight independent experiments.

either agent alone in three HCC cell lines (Fig. 1). We further used DAPI staining to determine apoptosis in HCC cells by a combination of anti-Fas agonistic antibody and these metabolic inhibitors (Fig. 2). Anti-Fas agonistic antibody alone failed to induce nuclear fragmentation or condensation (Fig. 2A). Nuclear morphologic exchanges were also not detected in ActD or CHX alone (data not shown); however, the combination of 100 ng/ml anti-Fas agonistic antibody and 0.5 μg/ml ActD or 2.0 μg/ml CHX showed typical apoptotic features (Fig. 2, B and C). These results showed that subtoxic levels of ActD or CHX could nullify resistance to Fas-induced apoptosis in these HCC cells.

Pro-caspase-8 and -3 Cleavage by Anti-Fas Agonistic Antibody–Induced Apoptosis

We speculated that apoptosis induction by the combination of anti-Fas agonistic antibody and metabolic inhibitors followed a cascade of caspase activation. To assess the caspase activity with respect to this apoptosis induction and to determine at what point HCC cell lines inhibit Fas-induced caspase cascade, we examined the expression of pro-caspase-8 and pro-caspase-3 (Fig. 3). Although anti-Fas agonistic antibody alone did not show a change of pro-caspase-8 and -3 expression levels, a combination of anti-Fas agonistic antibody and ActD or CHX simultaneously showed down-regulation of both pro-caspase-8 and -3 protein expression levels.

cFLIP Protein Expression in Human HCC Tissues and Cells

We showed that a combination of anti-Fas agonistic antibody and metabolic inhibitors induced both caspase-8 and caspase-3 activation in HCC cell lines (Fig. 3). This result suggests that apoptotic signal inhibition may exist upstream of caspase-8 and implicates cFLIP, which is an anti-apoptotic protein. To determine in situ expression of cFLIP, 20 human HCC tissues and 20 nontumor hepatic tissues were examined for cFLIP protein expression by immunohistochemistry (Fig. 4, A and B). Although both HCC and nontumor preparations showed the expression of cFLIP in their cytoplasmic area, staining was stronger in HCC than in nontumor hepatic cells. There was a statistically significant difference in cytoplasmic staining intensity between HCC and nontumor cells (Fig. 4C). There was no nuclear stain in either cell type.

Next we investigated the expression of cFLIP protein in seven HCC cells (HepG2, SK-Hep1, HLE, Hep3B, Huh7, Chang liver, and PLC/PRF/5). As shown in Figure 5, the expression of 55-kd cFLIP protein was detected in all seven HCC cells and Jurkat cells. Although we investigated the expression of cFLIPs, cFLIPs protein was not detected in all seven HCC cells (data not shown). Three HCC cell lines, including HepG2, SK-Hep1, and HLE, expressed higher levels of cFLIP than the other HCC cells. We also analyzed cFLIP protein expression in HCC cells (HepG2, SK-Hep1, and HLE), which were incubated with subtoxic levels of ActD (0.5 μM) or CHX (2.0 μM) for 24 hours. In this experiment it was seen that cFLIP expression decreased in HCC cells (Fig. 6).

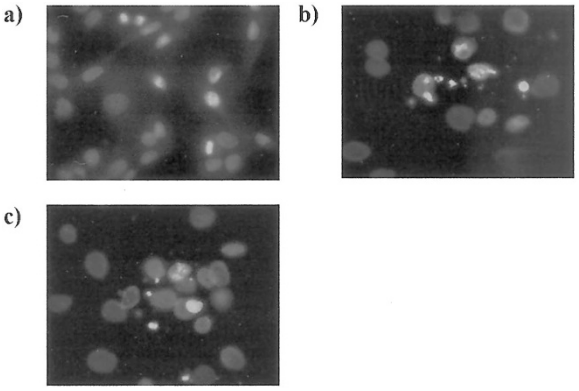

Figure 2.
DAPI (4'6-diamidino-2-phenylindol) staining of hepatocellular carcinoma (HCC) cells. (A) HCC cells (HLE) were incubated with 100 ng/ml anti-Fas antibody. (B) Cells were incubated with 100 ng/ml anti-Fas agonistic antibody and 0.5 μg/ml actinomycin D (ActD). (C) Cells were incubated with 100 ng/ml anti-Fas agonistic antibody and 2.0 μg/ml cycloheximide (CHX). Co-incubation of anti-Fas agonistic antibody and ActD or CHX demonstrated typical apoptotic features, including nuclear condensation and nuclear fragmentation.

cFLIP Down-Regulation Sensitized HCC Cells to Fas-Mediated Apoptosis

The observation that cFLIP protein is down-regulated by subtoxic levels of ActD or CHX and that apoptosis is induced by a combination of anti-Fas antibody and metabolic inhibitors suggests the importance of cFLIP in apoptotic inhibition. To evaluate the role of cFLIP protein in apoptosis in HCC cells, we made a cFLIP antisense oligodeoxynucleotide and transfected it in HLE cells. As shown in Figure 7A, cFLIP protein expression showed down-regulation after transfection of cFLIP antisense oligodeoxynucleotide, but cFLIP sense oligodeoxynucleotide did not affect the expression level of cFLIP. Pro-caspase-8 and -3 expression did not change upon cFLIP sense or antisense oligodeoxynucleotide transfection (data not shown).

Next, to assess the effect of cFLIP down-regulation by cFLIP antisense transfection on death-receptor stimulation in HLE cells, we analyzed cell viability. Under conditions of down-regulation of cFLIP expression, death-receptor stimulation (including anti-Fas agonistic antibody, TNF-α, or TRAIL) caused a decrease of cell viability compared with anti-Fas agonistic antibody, TNF-α, or TRAIL alone (Fig. 7B). In contrast, cell viability with cFLIP sense transfection was not affected by these stimulators.

cFLIP-Regulated TNF-α or TRAIL-Induced NF-κB Activation in HCC Cells

Human HCC cells showed strong resistance to TRAIL-mediated apoptosis and TRAIL-induced NF-κB activation in HCC cells in a dose-dependent manner (Yamanaka et al, 2000). However, the role of cFLIP in

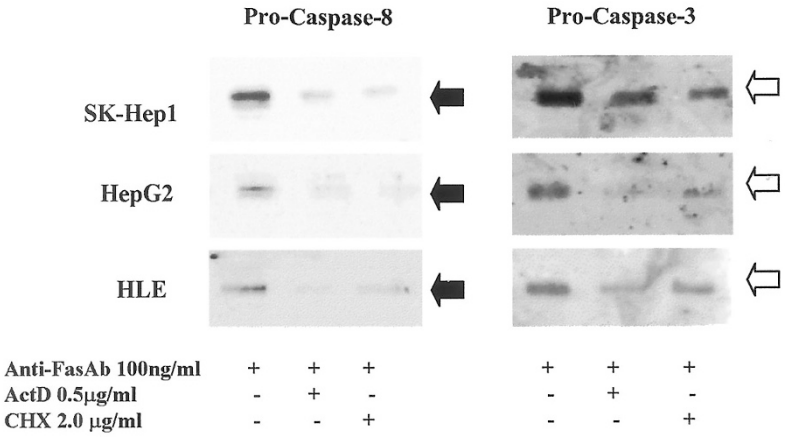

Figure 3.
Expression of pro-caspase-8 (55 kd) and -3 (32 kd) determined by Western blotting. For 24 hours, 2×10^5 hepatocellular carcinoma (SK-Hep1, HLE, and HepG2) cells were cultured in a 60-mm dish, followed by addition of 0.5 μg/ml actinomycin D (ActD) or 2.0 μg/ml cycloheximide (CHX) and 100 ng/ml anti-Fas agonistic antibody for 18 hours. *Closed arrows* indicate the expression of pro-caspase-8; *opened arrows* indicate pro-caspase-3.

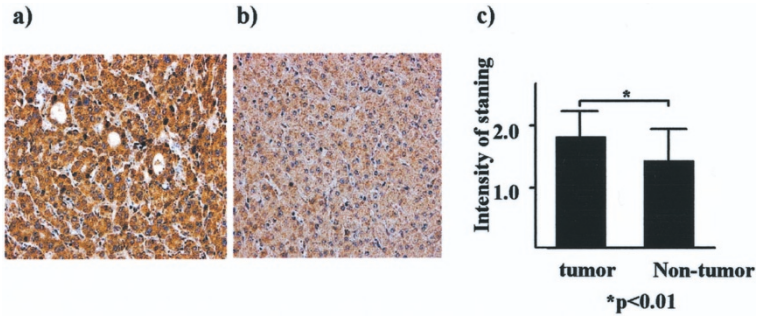

Figure 4.
Indirect immunohistochemical staining of human HCC (A) or nontumor hepatic cells (B) with cellular FLICE/caspase-8–inhibitory protein (cFLIP). Both preparations were stained with antibody for cFLIP and counterstained with hematoxylin (×200). (C) Comparison of intensity of staining between resected human hepatocellular carcinoma (HCC) tissue and non-HCC tissue. Data shown are the mean ± SD of 25 independent experiments. The Chi-square test was used to compare the staining intensity of HCC and nontumor hepatic cells. $p < 0.05$ was regarded as statistically significant.

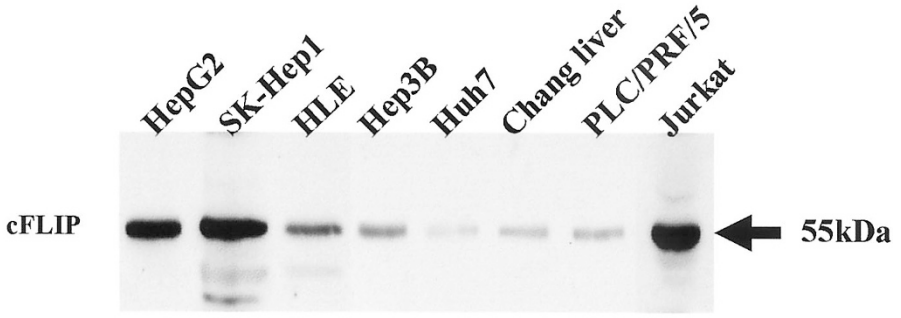

Figure 5.
Cellular FLICE/caspase-8–inhibitory protein (cFLIP) expression in seven human hepatocellular carcinoma cell lines (HepG2, HLE, SK-Hep1, Hep3B, Huh7, Chang liver, and PLC/PRF/5) and a human T cell lymphoma cell line (Jurkat) by Western blotting. *Arrow* indicates the expression of cFLIP (55 kd).

death-receptor–induced NF-κB activation in HCC cells is not clear. At first, we analyzed the NF-κB activation level of HCC cells with TNF-α or TRAIL stimulation under conditions of cFLIP up-regulation. HCC cells showed an increase of cFLIP expression level with cFLIP expression vector transfection, compared with a mock transfection (Fig. 8A). HCC cells with cFLIP up-regulation were stimulated with 50 ng/ml TNF-α or 50 ng/ml TRAIL for 12 hours. As shown in Figure 8B, we confirmed that NF-κB activity induced by TNF-α or TRAIL in these HCC cells substantially increased under cFLIP up-regulation.

cFLIP inhibits caspase activity by interfering with pro-caspase-8 cleavage, which is the starting point of the caspase-cascade and inhibits apoptosis (Irmler et al, 1997). We next investigated the effect on NF-κB

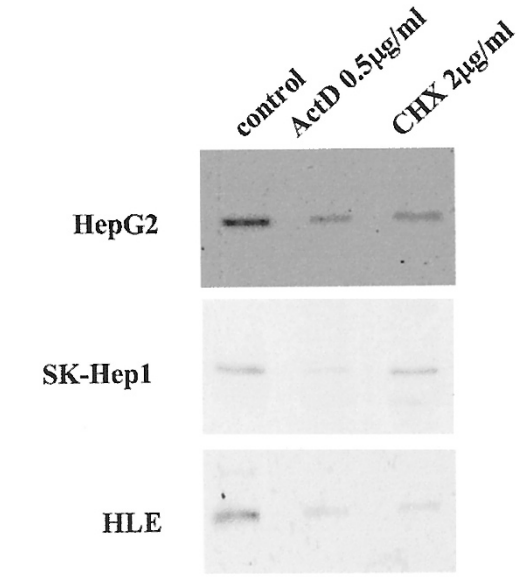

Figure 6.
Cellular FLICE/caspase-8–inhibitory protein (cFLIP) expression in hepatocellular carcinoma cells (SK-Hep1, HLE, and HepG2) after incubation with 0.5 μg/ml ActD or 2.0 μg/ml CHX for 24 hours. The cFLIP expression was analyzed by Western blotting.

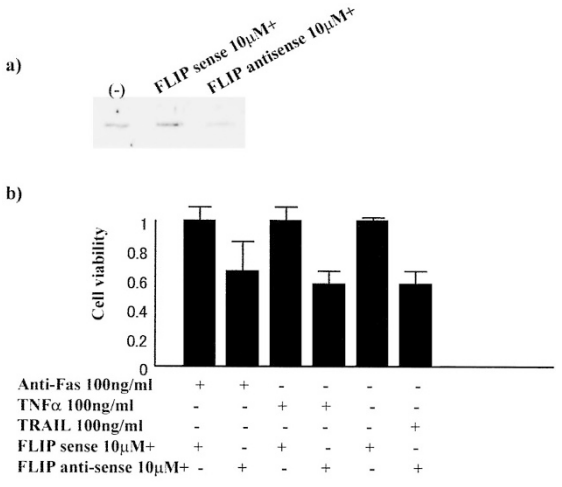

Figure 7.
(A) The down-regulation of cellular FLICE/caspase-8–inhibitory protein (cFLIP) expression in HLE cells after transfection of cFLIP antisense oligodeoxynucleotides. cFLIP sense oligodeoxynucleotides transfection did not affect cFLIP expression level. Hepatocellular carcinoma cells were transfected with 10 μM cFLIP sense or antisense oligodeoxynucleotides and incubated for 24 hours at 37 C°. (B) Cell viability decreased on incubation with 100 ng/ml anti-Fas agonistic antibody, 100 ng/ml TNF-α, or 100 ng/ml TNF-related apoptosis-induced ligand (TRAIL) after cFLIP antisense oligodeoxynucleotides transfection in HLE cells. cFLIP sense oligodeoxynucleotides transfection did not affect cell viability with anti-Fas agonistic antibody, TNF-α, or TRAIL incubation. Cell viability was assessed by MTT assay. Data shown are the mean $\pm$ SD of five independent experiments.

activation by pan-caspase-inhibitor, Z-VAD-fmk pretreatment with cFLIP down-regulation by cFLIP antisense oligodeoxynucleotides transfection. Pretreatment with 40 μM Z-VAD-fmk further increased TNF-α or TRAIL-induced NF-κB activation (Fig. 9A). In contrast to induction of NF-κB activation by cFLIP up-regulation, FLIP down-regulation attenuated the effect of TNF-α or TRAIL-induced NF-κB activation (Fig. 9B). However, NF-κB activation was restored by Z-VAD-fmk addition, though cFLIP protein was down-regulated (Fig. 9B). In these experiments cell viabilities did not change (data not shown).

cFLIP Protein Expression is Regulated through NF-κB Activation or PI-3/Akt Kinase Pathway in HCC Cells

Finally, we investigated cFLIP expression exchange in HCC cells by NF-κB or PI-3/Akt kinase pathway stimulation. Stimulation with 50 ng/ml TNF-α or TRAIL, which has already been shown to activate NF-κB, showed up-regulation of cFLIP in HLE cells (Fig. 10). The up-regulation of cFLIP in HLE cells was also observed after 100 ng/ml vascular endothelial growth factor (VEGF) stimulation (Fig. 10). However, treatment with 200 nM wortmannin, which inhibits the PI-3/Akt kinase pathway, showed a down-regulation pf cFLIP in HLE cells (Fig. 10). These reagents did not affect cell viability (data not shown). Hence, it is implicated that cFLIP expression level is regulated through the NF-κB or PI-3/Akt kinase pathways in HCC cells.

Discussion

TNF receptor family–mediated apoptosis in a variety of human cells plays an important role in the host's defense against tumor cells via T lymphocytes or macrophages. These receptors contain intracellular regions called "death domains," which convert direct receptor-triggered signaling into apoptosis (Nagata, 1997). To escape the host's immune response, it is necessary for tumor cells to be more resistant to this TNF family–induced apoptosis. In fact, most HCC cells show strong resistance to Fas (Fulda et al, 2000; Natoli et al, 1995) and TRAIL receptor–mediated apoptosis (Yamanaka et al, 2000), and have several mechanisms, such as down-regulation of death-signaling component (Fujikawa et al, 2000; Strand et al, 1996) or expression of antiapoptotic proteins (Ito et al, 2000; Shiraki et al, 2002; Soini et al, 1996; Takehara et al, 2001).

The current study demonstrated that the resistance of HCC cells to Fas-mediated apoptosis can be overcome by a subtoxic level of ActD that inhibits messenger RNA transcription and CHX that inhibits protein synthesis. The same results were obtained in TRAIL-R–mediated apoptosis (Yamanaka et al, 2000). These data suggest that resistance to apoptosis was more likely mediated by intracellular signaling events than by alternation in receptor expression or the presence of decoy receptors and that the components including cFLIP of Fas-mediated apoptosis exist in these HCC cells. Then it becomes crucial to determine the critical endogenous suppressors of Fas-mediated apoptosis that are sensitive to those metabolic inhibitors.

To address these questions, it is important to determine at which level HCC cells inhibit TNF family–induced death signaling. First, we have examined processing of the most upstream protease,

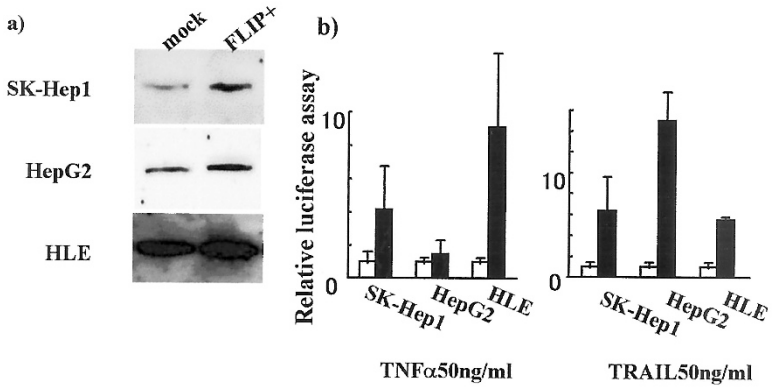

Figure 8.

(A) The up-regulation of cellular FLICE/caspase-8–inhibitory protein (cFLIP) expression by cFLIP expression vector transfection in HLE cells. For 24 hours 2×10^5 HCC cells were grown in a 60-mm dish at 37° C the day before transfection. The cFLIP expression vector or mock vector was transfected using FuGENE 6 for 24 hours at 37° C. Western blotting showed an increase of cFLIP expression in cFLIP expression vector transfected cells, more than in control vector transfected cells. (B) Effects of cFLIP up-regulation on NF-κB activation with TNF-α or TNF-related apoptosis-induced ligand (TRAIL) treatment in hepatocellular carcinoma (HCC) cells. *Open bars* show NF-κB activation with mock-transfected cells, and *closed bars* show NF-κB activation with cFLIP expression vector-transfected cells. The pNF-κB-Luc Vector and cFLIP expression vector or mock vector were co-transfected in human HCC cells. After incubation for 24 hours at 37 C°, cells were stimulated by 50 ng/ml TNF-α or 50 ng/ml TRAIL for 12 hours. NF-κB activation was assessed by luciferase assay system. Data shown are the mean ± SD of three independent experiments.

caspase-8, and a critical down-stream protease, caspase-3, in the protease cascade, when Fas receptor was stimulated in the presence or absence of the metabolic inhibitors. Our results show that caspase-8 was not activated by cleavage, and in addition caspase-3 was not cleaved when treated with anti-Fas agonistic antibody either. However, both caspases were significantly processed in the presence of the metabolic inhibitors when treated with anti-Fas agonistic antibody. These results indicate that the Fas-mediated death signal was inhibited at the caspase-8 level, and inhibitors of caspase-8 may play a critical role in resistance to death-receptor–mediated apoptosis. We have obtained similar results, which indicate that TRAIL-R–mediated apoptosis is also inhibited at both caspase-8 and caspase-3 levels (Yamanaka et al, 2000).

cFLIP contains tandem death-effector domains and caspase-like domain similar to procaspase-8 but lacks amino acid residues that are critical for caspase activity, most notably the cysteine in the catalytic center (Irmler et al, 1997). cFLIP suppresses all known receptor-mediated apoptosis by inhibiting procaspase-8 processing (Irmler et al, 1997). Surprisingly, some viruses encoded the homolog of cFLIP, which also controls sensitivity toward death-receptor–mediated apoptosis (Thome et al, 1997). Also, cFLIP-deficient mice do not survive past Day 10.5 of embryogenesis and exhibit impaired heart development (Yeh et al, 2000). These findings also suggested that cFLIP has a crucial role in regulation of cell death and a potential role in malignant transformation. Therefore, we have investigated the expression and function of caspase-8 inhibitor, cFLIP, in HCC cells.

Our study shows that cFLIPs are constitutively expressed in all HCC cell lines examined and that the expression level of cFLIP depended on the individual cell type. During apoptosis induced by anti-Fas agonistic antibody in the presence of metabolic inhibitors, cFLIP was significantly down-regulated, indicating that cFLIP was one of the sensitive targets of protein synthesis inhibitors for the sensitization of apoptosis.

We then investigated the antiapoptotic function of cFLIP in HCC cells. Experiments using cFLIP antisense oligonucleotides revealed that specific down-regulation of cFLIP sensitized about 30% to 50% of HLE cells to all Fas, TNF-R, and TRAIL-R–mediated apoptosis. These results support the crucial role of cFLIP in death-receptor–mediated apoptosis and also other antiapoptotic mechanisms that exist in HCC cells. In fact, inhibitors of apoptosis family or Bcl family are also important apoptotic inhibitors overexpressed in HCC cells (Takehara et al, 2001). A recent study revealed that the relative levels of caspase-8 and cFLIP are an important determinant of susceptibility to Fas-mediated apoptosis in malignant cells (Krueger et al, 2001; Tepper and Seldin, 1999), although it is not clear what amounts of cFLIP are required to protect cells from apoptosis. It is likely that high levels of cFLIP might contribute to antiapoptotic function.

In human HCC tissues, cFLIP was expressed significantly stronger in HCC cells than surrounding non-tumor tissues. In colonic adenocarcinomas, cFLIP is frequently elevated at both mRNA level and protein level (Ryu et al, 2001), and is also strongly expressed in 83% of melanomas (Bullani et al, 2001), although it is not expressed in most benign melanocytic lesions. In human melanoma cells, the expression level of cFLIP correlated with resistance to TRAIL-induced apoptosis (Griffith and Lynch, 1998). Furthermore, overexpression of cFLIP by transfection in a Fas- and TRAIL-sensitive human melanoma cell line rendered this cell line more resistant to stimulation by both FasL and TRAIL (Bullani et al, 2001). This also suggests that cell-death inhibition by cFLIP overexpression might be the fundamental mechanism of cell-death regulation in malignant transformed cells. It has also been reported that vFLIP and cFLIP mediate the immune escape of tumors (Djerbi et al, 1999; Medema et al, 1999). A

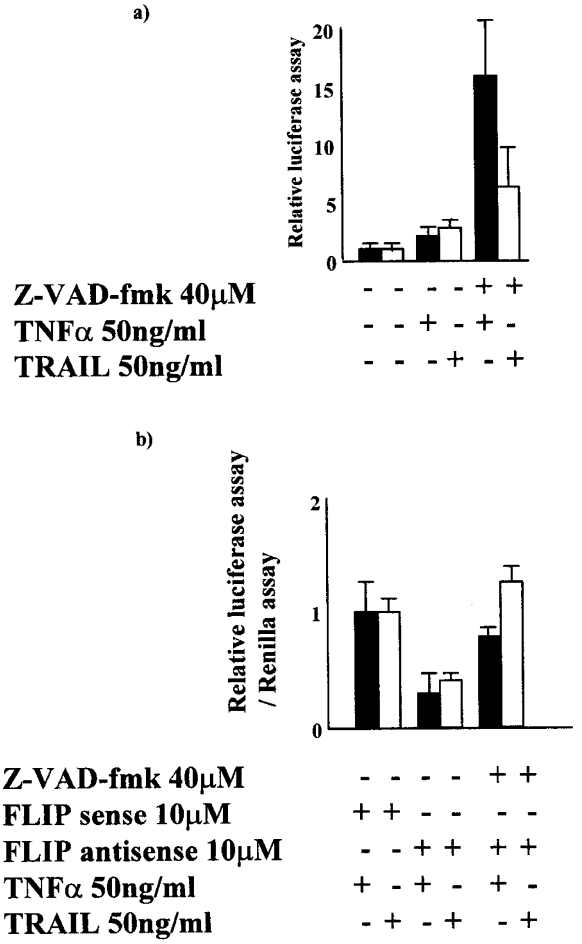

Figure 9.

Effects of Z-VAD-fmk treatment or cellular FLICE/caspase-8–inhibitory protein (cFLIP) down-regulation by 10 μM cFLIP antisense oligodeoxynucleotides transfection on NF-κB activation with TNF-α or TNF-related apoptosis-induced ligand (TRAIL) stimulation in HLE cells. *Closed bars* show 50 ng/ml TNF-α stimulation, and *open bars* show 50 ng/ml TRAIL stimulation. The shown data are the mean $\pm$ SD of three independent experiments. (A) HLE cells were pretreated with 40 μM Z-VAD-fmk for 1 hour before treatment with TNF-α or TRAIL. Reporter gene activity was measured 12 hours after treatment. Z-VAD-fmk treatment increased NF-κB activation by TNF-α or TRAIL stimulation more than TNF-α or TRAIL alone. (B) The pNF-κB-Luc Vector and 10 μM cFLIP antisense or sense oligodeoxynucleotides were co-transfected in HLE cells. After incubation for 24 hours at 37 C°, cells were stimulated by 50 ng/ml TNF-α alone or 50 ng/ml TNF-α with 40 μM Z-VAD-fmk for 1 hour pretreatment. TRAIL was also used under the same conditions. Reporter gene assay was measured after 4 hours of TNF-α or TRAIL treatment. cFLIP down-regulation attenuated NF-κB activation by TNF-α or TRAIL stimulation. The effects of cFLIP down-regulation were restored with 40 μM Z-VAD-fmk incubation.

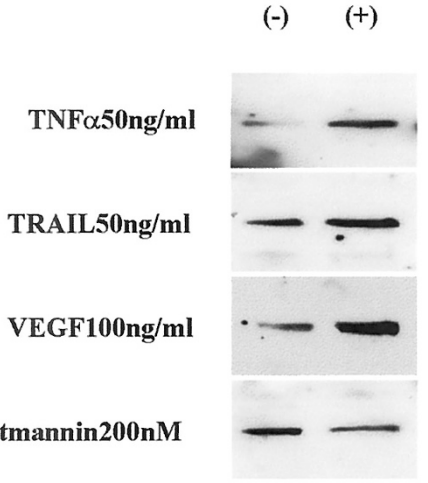

Figure 10.

Effects of TNF-α, TNF-related apoptosis-induced ligand (TRAIL), vascular endothelial growth factor (VEGF), or wortmannin (WM) treatment on cellular FLICE/caspase-8–inhibitory protein (cFLIP) expression in HLE cells. HLE cells were incubated with these reagents (50 ng/ml TNF-α, 50 ng/ml TRAIL, 100 ng/ml VEGF, or 200 nM WM) for 24 hours and analyzed by Western blotting. These results showed that cFLIP expression was up-regulated by TNF-α, TRAIL, or VEGF treatment and down-regulated by WM.

recent study has demonstrated that, using in vivo murine tumor models, cFLIP does help in escape from T cell immunity and that tumor cells are selected for elevated cFLIP expression (Medema et al, 1999). Thus, we hypothesized that human HCC cells with elevated cFLIP expression might also be selected for survival, most likely due to immunological pressure exerted by the host during carcinogenesis and tumor development.

NF-κB is a transcription factor that prevents cell death and promotes cell survival, regulating several gene encoding proteins such as cellular inhibitor of apoptosis (Wang et al, 1998), TNF-R associated factor 1 (TRAF1) (Wang et al, 1998), and Bcl-xL (Khoshnan et al, 2000).

NF-κB is known to be activated by receptor signals such as TNF-R1, TRAIL-R1, TRAIL-R2 (Ponton et al, 1996; Wajant et al, 2000), IL-1β (Barnes and Karin, 1997), or IL-18 (Robinson et al, 1997). The death-domain–containing receptors TNF-R1, TRAIL-R1, and -R2 are capable of initiating apoptosis but also have gene-induced property via NF-κB. We have recently demonstrated that TRAIL-R1 and -R2 expression was prevalent and that TRAIL activated NF-κB rather than induced apoptosis in HCC cells (Yamanaka et al, 2000). These pieces of evidence suggested that HCC cells use TRAIL signaling as their cell survival signal. Then it becomes important to elucidate how these transformed cells regulate the death-receptor–mediated signaling to survive.

In this study we have demonstrated that cFLIP overexpression or pan-caspase-inhibitor, Z-VAD-fmk, accelerated NF-κB activation induced by TNF-R or TRAIL-R stimulation. In addition, cFLIP suppression by antisense oligonucleotides attenuated the NF-κB activation. These results imply that cFLIP protein plays the important role of an endogenous signaling pathway switching modulator to either apoptosis induction or cell proliferation. It has been reported that cFLIP interacts with TRAF1 and TRAF2, as well as with the receptor interacting protein (RIP), resulting in NF-κB activation in Jurkat cells (Kataoka et al, 2000). In addition, a recent study showed that caspase-mediated cleavage of RIP inhibits its capacity to activate NF-κB (Harper et al, 2001) and that cFLIP may prevent RIP cleavage by inhibiting these caspases and enhance NF-κB. Our study clearly demonstrates that

NF-κB activation was restored by Z-VAD-fmk even when cFLIP is down-regulated. This will also support the previous findings. In contrast to our study, there is a report that the overexpression of cFLIP inhibits death-receptor–mediated NF-κB activation in HeLa cells (Wajant et al, 2000). These differences may be due to cell types or other possible candidates that can be part of the receptor-signaling complex of death receptors. Further study will be needed to show how cFLIP regulates NF-κB activation. In this study, we provide evidence to show that cFLIP is not simply an inhibitor of death-receptor–mediated apoptosis, but it also mediates the activation of NF-κB, which promotes cell survival in HCC.

How is cFLIP expression regulated in HCC cells? Our study shows that either NF-κB or PI-3/Akt kinase activation can induce FLIP protein up-regulation and PI-3/Akt kinase inhibition can induce FLIP protein down-regulation in HLE cells. NF-κB or PI-3/Akt kinase are associated with the regulation of expression of many genes and play an important role in oncogenesis or tumor cell growth (Rayet and Gelinas, 1999; Shi et al, 2002). Furthermore, metabolic inhibitors acting on either transcription or translation were shown to abolish cFLIP expression. Our results support previous findings concerning cFLIP regulation using other transformed cell lines. In addition, autocrine regulation of cFLIP may exist, because HCC cells strongly expresses VEGF (Chow et al, 1997; Yamaguchi et al, 1998) by themselves. Furthermore, immunological cytotoxicity of lymphocytes by the TNF family may contribute to cFLIP up-regulation, because TNF-receptor families including TNF-R1 and -R2, and TRAIL-R1, -R2, -R3, or -R4 have signaling pathways that involve NF-κB activation (Bernard et al, 2001; Degli-Esposti et al, 1997; Ponton et al, 1996; Seitz et al, 2001; Wajant et al, 2000). The pieces of evidence indicate that cFLIP regulation is tightly controlled by many factors both endogenously and exogenously.

In conclusion we have demonstrated that cFLIP is not simply an inhibitor of death-receptor–mediated apoptosis but that it also induced NF-κB activation. These results indicate that cFLIP plays an important role in controlling cell death or survival and switching signals to either a caspase cascade or NF-κB. Our study implies that a strategy to inhibit cFLIP expression may be a potential tool in the useful treatment of human HCCs.

Materials and Methods

Cell Lines and Human HCC Tissues

The Jurkat T cell line, the human HCC cell lines, HepG2, Hep3B, and SK-Hep1 cells were purchased from American Type Culture Collection (Rockville, Maryland). The HCC cell lines, Huh7, HLE, and PLC/PRF/5 were all purchased from the Health Science Research Resource Bank (Osaka, Japan). The Jurkat cells were cultured in RPM1 1640 (Gibco BRL, Grand Island, New York). The other cell lines were cultured in Dulbecco's modified Eagle medium (Dainippon Pharmaceutical Company, Ltd., Osaka, Japan). All media were supplemented with 1% penicillin/streptomycin (Gibco BRL) and 10% heat-inactivated FCS (Gibco BRL). Human HCC tissues and nontumor tissues were obtained from surgical resection for immunohistochemical analysis. We obtained informed consent from patients for subsequent use of their resected tissues.

Assessment of Viability of HCC Cells

To assess the viability of HCC cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. The HCC cells were plated at a density of 5×10^3 cells/well in 96 well microtiter plates (Corning Glass Works, Corning, New York). Either 0.5 μg/mL ActD (Sigma Chemical Company, St. Louis, Missouri) or 2.0 μg/mL CHX (Sigma) was added in the absence or presence of 100 ng/ml anti-Fas agonistic antibody (MBL, Nagoya, Japan), and the plate was incubated for 24 hours, respectively. The live-cell count was determined using a Cell Titer 96 assay kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. The absorbance of each well was measured at 570 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, California).

Detection of Apoptosis

For 24 hours 2×10^4 HCC cells/well were cultured in an 8-well Lab-tek II chamber slide (NUNC™ Brand Products, Demmark), followed by the addition of 0.5 μg/mL ActD (Sigma) or 2.0 μg/mL CHX (Sigma) and 100 ng/ml anti-Fas agonistic antibody (MBL). After a further incubation for 24 hours, cell nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI) (Sigma) and observed with a fluorescence microscope (Zeiss, Göttingen, Germany).

Western Blotting

Expression of pro-caspase-8 and -3, $cFLIP_L$, and $cFLIP_S$ were analyzed by Western blotting. Anti–caspase-8 was purchased from Upstate Biotechnology (Lake Placid, New York). Caspase-3 p20 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). These antibodies did not detect active forms of caspase-8 or caspase-3. Anti-FLIP polyclonal antibody was purchased from Millennium Biotechnology (Romona, California). Anti-$FLIP_{(\gamma/\delta)}$ were purchased from Calbiochem (Darmstadt, Germany). HCC cells were harvested and lysed in lysis buffer (50 mmol/L Tris-HCl, 8 pH, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride) on ice. Protein contents measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein from each extract were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Toyo Roshi, Tokyo, Japan). Blots were blocked by incubation in 5% nonfat dried milk in Tris buffered

saline overnight at 4° C and probed for 2 hours at room temperature with primary antibody. The immunoblots were then probed with horseradish peroxidase-conjugated Ig G (1:2000 diluted with 5% nonfat dried milk in Tris-HCl; 7.5 pH and 0.05% Tween 20). Signal was detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Immunohistochemical Staining for cFLIP Protein

Immunohistochemical staining for cFLIP protein was performed on resected HCC tissues and nontumor tissues. Deparaffinized sections were heated for 10 minutes at 120° C in a pressure cooker to reactivate antigen and treated with 0.3% H_2O_2 in methanol for 20 minutes to abolish endogenous peroxidase activity. Sections were blocked with normal goat serum in PBS (PBS) and incubated overnight with a 1:40 dilution (in PBS) of anti-cFLIP polyclonal antibody at 4° C. The sections were incubated with a second biotinylated antirabbit Ig diluted 1:200 in PBS, followed by a 1:200 dilution of avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, California) diluted 1:200 in PBS for 30 minutes at room temperature. They were developed in a substrate solution of 0.01% 3,3'- diaminobenzidine-hydrogen peroxide for visualization and counterstained with Mayer's hematoxylin. The stained sections were examined under ×200 magnification. The immunostaining was analyzed semiquantitatively using a scoring system: 1 was defined as weak staining intensity, 2 was defined as moderate staining intensity, and 3 was defined as strong staining intensity. The results presented for each tissue are representative of immunohistochemical analysis of multiple immunostained slides.

cFLIP Antisense Oligodeoxynucleotides or cFLIP Expression Vector Transfection in HCC Cells

To inhibit cFLIP protein expression in HCC cells, phosphorothiorate antisense oligodeoxynucleotides to inhibit the cFLIP initiation codons and control sense oligodeoxynucleotides with the following published sequences were used (Perlman et al, 1999): cFLIP antisense, 5'-gatttcagcagacatcctac-3'; and cFLIP sense, 5'-catcctacagacgacttcag-3'. Using FuGENE 6 system (Boehringer Mannheim, Mannheim, Germany), 1×10^5 HCC cells/well were transfected with 10 μM cFLIP antisense or sense oligodeoxynucleotides according to the manufacture's protocol.

The cFLIP expression vector was a gift from Dr. Valentina Screpanti (Department of Immunology, University of Stockholm, Sweden). The cFLIP expression vector was transfected using FuGENE 6 (Boehringer Mannheim), essentially according to the manufacture's protocol, for 24 hours at 37° C. As a mock vector, pcDNA3.1 (−)/Myc-His (Invitrogen, Carlsbad, California) was used. This vector was transfected using similar conditions of cFLIP transfection. Expression of cFLIP protein was analyzed by Western blotting.

NF-κB Luciferase Reporter Gene Assay

The pNF-κB-Luc Vector (Mercury Pathway Profiling System) was obtained from Clontech (San Diego, California). Using FuGENE 6 (Boehringer Mannheim), 2×10^5 human HCC cells were transfected according to the manufacturer's protocol. Luciferase activity was determined from cell extracts by means of a luciferase assay system (Promega) and luminometer (Berthold Analytical Instruction).

Renilla Luciferase Assay

The phRL-TK vector was purchased from Promega Corporation (Madison, Wisconsin). Two μg phRL-TK vector and 3 μg pNF-κB-Luc Vector (Mercury Pathway Profiling System) were transfected with 10 μM cFLIP antisense or 10 μM cFLIP sense oligodeoxynucleotides using FuGENE 6 (Boehringer Mannheim), essentially according to the manufacture's protocol, for 24 hours at 37° C. Renilla luciferase activity was determined from cell extracts by means of a renilla luciferase assay system (Promega) and luminometer (Berthold Analytical Instruction). The value of NF-κB luciferase activities were modulated by the value of renilla luciferase activities.

HCC Cell Stimulation by NF-κB Activation or Phosphatidylinostiol-3 (PI-3)/Akt Kinase Activation

To analyze the effect for cFLIP protein expression in HCC cells mediated by the NF-κB activation pathway, 2×10^5 HCC cells were incubated with 50 ng/ml TRAIL (R&D Systems) or 50 ng/ml TNF-α (Cosmo Bio) for 24 hours. With respect to PI-3/Akt kinase pathway, we used 100 ng/ml recombinant human VEGF (Peprotech EC Ltd., London, United Kingdom) or 200 nM wortmannin (SIGMA). Expression of cFLIP protein was analyzed by Western blotting.

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