Cell Death Induction by CTL: Perforin/Granzyme B System Dominantly Acts for Cell Death Induction in Human Hepatocellular Carcinoma Cells (44563)

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Abstract. **Cell death induction by cytotoxic T lymphocytes (CTLs) is an important thesis for the understanding of tumor immunotherapy. In the current study we investigated the molecular machinery of CTL-induced cell death in human hepatocellular carcinoma cell lines (HCC lines). CTLs prepared from human peripheral blood induced cell death in all tested HCC lines. As the CTL-induced death system, the effectiveness of Fas ligand/Fas and/or Perforin/Granzyme B systems has been suggested, whereas cell death induction by CTLs was shown independently on Fas expression in the current study. Using various tetrapeptide inhibitors for caspase and its associated factor, we additionally demonstrated that inhibitors for caspase 3 (Ac-DEVD-CHO) and caspase 8/granzyme B (Ac-IETD-CHO) suppressed CTL-induced cell death, but an inhibitor for Fas-activated serine proteinase, which acts for the caspase 3 activator, did not, suggesting that CTL-induced cell death was initiated by the Perforin/Granzyme B system, rather than the Fas ligand/Fas system. On the basis of our current results, we report here that the Perforin/Granzyme B system acts dominantly for the cell death induction of HCC lines.** [P.S.E.B.M. 2000, Vol 225:143–150]

Cell death is an essential phenomenon for cell homeostasis, as well as cell growth, and has been well documented during embryonic and postembryonic development (1, 2). The two distinct processes leading to cell death are apoptotic cell death and necrotic cell death (1). Apoptotic cell death is accompanied by the condensation and/or fragmentation of nuclei, apoptotic body formation, and chromosomal DNA fragmentation into 180-base-pair oligomers (1). Multiple studies have demonstrated the important role of apoptotic cell death in various disease states and physiological cell death (3–5), and many factors involved with the death signaling have been identified.

Fas, the type I transmembrane protein belonging to the nerve growth factor/tumor necrosis factor (TNF) receptor family, transduces the cell death signaling upon stimulation of the Fas ligand (4, 6, 7). Fas-stimulated, cell death signaling is mediated by caspase, which is the nomenclature of the interleukin-1β converting enzyme (ICE)/CED-3 cysteine proteinase family member (2, 8). Presently, 14 genes have been identified as the caspase family member and three subfamilies referred to as ICE-, CPP32- and ICH-1 subfamilies (8). Among caspase family members, the CPP32 subfamily, especially caspase 3 (CPP32/Yama/Apopain) (9, 10) is the most important factor for Fas-initiated cell death signaling (11–14). Caspase 3 activation is induced by various factors, such as cytoplasmic serine proteinase cleaving at the p3 site (15), caspase 8 cleaving at the p17 site (FLICE/MACH) (15–18), and cytotoxic T lymphocyte (CTL)-derived serine proteinase Granzyme B (18, 19). Thus, the most important factor for the cell death induction, caspase 3, is activated by endogenous and exogenous factors.

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Viral hepatitis is one of the CTL-associated disease states (20). CTLs recognize the viral antigen, such as the Hepatitis B viral antigen HBcAg, expressed on the cell surface of infected hepatocytes, and induce the cell death signaling (20) that induces cell death. Two hypothesized molecular mechanisms have been suggested as the CTL-induced cell death pathway: One is the Fas ligand/Fas system and another is the Perforin/Granzyme B system (21). Both systems induce cell death directly in target cells *via* caspase activation; however, the detailed machinery has yet to be elucidated. In the current study we investigated the molecular machinery of CTL-induced cell death as the *in vitro* model for viral hepatitis using human hepatocellular carcinoma cell lines, and we suggest that Perforin/Granzyme B system acts dominantly during the CTL-induced hepatocyte cell death, rather than the Fas ligand/Fas system.

Materials and Methods

Cell Lines and Culture. Human hepatocellular carcinoma lines, HLE, HLF, Huh7, SK-Hep1, Chang Liver, and Hep3B, were maintained in Dulbecco's Modified Eagle Medium (DMEM: Gibco BRL., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT) and 1% nonessential amino acid (NEAA: Cosmo Bio., Tokyo, Japan) in a humidified atmosphere of 5% CO_2 and 95% air.

Antibodies and Peptides. Agonistic Fas antibody (CH-11 clone) (22) and FITC-conjugated Fas antibody (UB2 clone) were purchased from Medical and Biological Laboratory (Nagoya, Japan). Monoclonal antibody for human MHC class I was purchased from DAKO (Kyoto, Japan). Concanamysin A (CMA) was purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Ac-IETD-CHO and Ac-DEVD-CHO were purchased from Peptide Laboratory (Osaka, Japan), and Ac-ESMD-CHO was synthesized at Asahi Techno Glass, Co., Ltd., (Tokyo, Japan).

Assay of Cell Viability. Cell viability was measured with the MTT assay as described previously (23). After treatment, 10 μl of PBS-diluted MTT (5 mg/ml) were added to each well, and the plate was incubated for 4 hr, followed by the addition of DMSO. The plate was maintained at room temperature for 5 min, and then read on a microtiter plate reader (Titerteck, Fukuoka, Japan) at A570/A690.

Cell viability of each group was also assessed by the Hoechst 33342 staining procedure as previously described (11) with some modifications (15, 24). Hoechst 33342 was purchased from Molecular Probes, Inc. (Eugene, OR). After treatment, cells were collected and stained with Hoechst 33342 and then observed by fluorescence microscopy. Cell viability was indicated by the ratio of cells carrying intact nuclei to total cells (about 5000 cells).

Preparation of Cell Extracts and Assay of Enzyme. Preparation of cell extracts and assay of enzyme was performed as previously described (11) with some modifications (15, 24). Cells were collected, washed with PBS, and suspended in PBS-EDTA (pH 7.4). After the addition of 10 μ*M* digitonin (Sigma), cells were incubated at 37°C for 10 min. Lysates were collected by centrifugation (15,000 rpm/5 min), and total protein concentration was determined using a DC protein assay kit (Bio Rad, Hercules, CA). For enzyme assay, aliquots were incubated with 10 μl DEVD-MCA (50 μ*M*), and the release of amino-4-methylcoumarin was monitored with a spectrofluorometer. One unit was defined as the amount of enzyme required to release 0.22 nmol AMC per min at 37°C.

Protein Extraction. Proteins were prepared for immunoblotting and immunoprecipitation analysis by lysis of cells with 1% NP-40 containing buffer for 30 min. All procedures were carried out at 4°C. Proteins were collected by centrifugation at 15,000 rpm for 15 min. Protein concentrations were determined by a method described previously (25, 26) using a DC protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Immunoblotting Analysis. Sample proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes by a semidry blotting system. The membranes 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 (Sigma, Tween-PBS), and then incubated overnight at room temperature with antibody diluted with PBS. After washing with Tween-PBS, the membranes 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-HRP (Vector Lab, Burlingame, CA) at room temperature for 1 hr. The membranes were washed with Tween-PBS and then developed with the ECL system (Amersham, Buckinghamshire, UK).

Preparation of Cytotoxic T Lymphocytes. Human PBMC separation from the peripheral blood of healthy individuals with the Ficoll/Hypaque method and CTL induction were performed as the previously described method (27) with some modifications. CTL population was generated in RHAMα medium (28) containing 5% autologous plasma or, when indicated, 5% plasma protein fraction with IL-1 (167 U/ml), IL-2 (67 U/ml), IL-4 (67 U/ml), and IL-6 (134 U/ml). CTLs were restimulated every 2weeks with target cells.

Cell Killing Assay. Cell killing assay of CTLs was assessed by crystal violet staining as described previously (27) with some modifications. Target cells were seeded in a 96-well plate, and then CTLs were added to each well at various E/T ratios after 12 hr. After the reaction of target cells with CTLs, each well was washed with PBS to remove CTLs, and adherent target cells were fixed and stained with crystal violet. The plate was washed and dried at room temperature. To each well, 80% methanol was added and the A570 in each well was measured, and then the survival

rate in each experiment was calculated as previously described (29).

Microinjection Analysis. Microinjection analysis was performed for the investigation of Granzyme B effect in HCC lines. Granzyme B was 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 sec. In the present study, no abnormalities were observed during the first week after the cells were microinjected with PBS.

Results

Expression and Death Induction Ability of Fas in HCC Lines. To investigate the death induction machinery of CTLs in the HCC line, we first examined the expression and death induction ability of Fas. Flow cytometry analysis using FITC-conjugated antibody for human Fas (UB2 clone) showed an intense expression of Fas in HLE, SK-Hep1, and Chang Liver cells, but not in HLF, Huh7, and Hep3B cells (Fig. 1A). In addition, any significant differences in the Fas expression level were not shown among Fas-positive HCC lines (Fig. 1A).

Further, we investigated the death induction ability of Fas in HCC lines using agonistic antibody for human Fas (CH-11 clone). When HCC lines were treated with CH-11 clone in the absence of *de novo* protein synthesis inhibitor actinomycin D, all HCC lines did not show the Fas-mediated cell death (Figs. 1B & 1C). In contrast, all Fas-expressing HCC lines (HLE, SK-Hep1, and Chang Liver) showed the Fas-mediated cell death in the presence of actinomycin D (Figs. 1B & 1C). These results indicate that all tested Fas-expressing HCC lines carry death factors that are necessary for the Fas-mediated cell death.

Expression and Activation of Caspase 3 in HCC Lines. Since caspase 3 is an essential factor for hepatitis (30), we investigated the caspase 3 expression and activation during Fas-mediated cell death in HCC lines. An expression of procaspase 3 was detected in all HCC lines at 32 kDa before co-treatment of CH-11 clone and actinomycin D. Above Fas-sensitive HCC lines, HLE, SK-Hep1, and Chang Liver cells showed the molecular shift to active form (17 kDa) and DEVD-MCA cleavage, used as a measurement of caspase 3 activity (11) by the Fas-initiated cell death signaling. However, Fas-insensitive HCC lines did not show either the molecular shift to active form or DEVD-MCA cleavage (Figs. 2A & 2B).

Characterization of Fas- and Granzyme B-Mediated Cell Death in HCC Lines. To characterize death signaling pathways of Fas- and Granzyme B-mediated cell death, tetrapeptide inhibitor was used for the experiments. In the current study, three tetrapeptide inhibitors were used: Ac-DEVD-CHO for caspase 3 (10, 11), Ac-IETD-CHO for caspase 8 and Granzyme B (18), and Ac-ESMD-CHO for caspase 3-activating serine proteinase (15). When Fas-positive HCC lines were reacted with the CH-11 clone, pretreatment with Ac-DEVD-CHO, Ac-IETD-CHO, or Ac-ESMD-CHO rescued cells from Fas-mediated cell death (Fig. 3A).

The Granzyme B (GzB)-induced HCC cell death was investigated. When all HCC lines were microinjected with the purified GzB, apoptotic cell death fashions (cell membrane blebbing, cell body atrophy, and nuclear condensation) were observed (Fig. 3B). In addition, immunoblotting analysis revealed that GzB injection induced the caspase 3 activation (the molecular shift of 32 kDa zymogen to 17 kDa active form). These results indicate that GzB induces apoptotic cell death as a result of caspase 3 activation.

Further, we investigated effects of tetrapeptide inhibitors in GzB-mediated cell death. When all HCC lines were microinjected with purified GzB, GzB-mediated cell death was detected (Fig. 3C). Interestingly, GzB-mediated cell death was prevented by pretreatment of Ac-DEVD-CHO and Ac-IETD-CHO, but not by Ac-ESMD-CHO (Fig. 3C).

The Molecular Machinery of CTL-Induced Cell Death. To investigate the molecular machinery of CTL-induced cell death in HCC lines, CTLs for two HCC lines (Fas-positive line: HLE; Fas-negative line: HLF) were prepared. CTLs used for the experiment were predominantly $CD3^+CD8^+$ T cells (HLE-CTL: 90%, HLF-CTL: 80%, unpublished data), and were MHC class I restricted (Figs. 5A & 5B). In the current study, each induced CTL showed the specific reaction to targeted cells; HLE-CTLs induced cell death in HLE, but not in HLF, and its contrary results were observed (unpublished data). As shown in Figure 4, induced CTLs showed the cell death induction in both targeted HCC lines, suggesting no involvement of Fas in CTL-induced cell death. In the current study, other HCC lines also show the same result as HLE and HLF cells.

Further, we investigated effects of tetrapeptide inhibitors and CMA that inhibits perforin-dependent cytotoxic activity (31, 32) in CTL-induced cell death. When HLE and HLF cells were reacted with each of the induced CTLs, pretreatment with Ac-DEVD-CHO, Ac-IETD-CHO, and CMA suppressed CTL-induced cell death, but Ac-ESMD-CHO did not (Figs. 5C & 5D).

Discussion

Recent studies have investigated the molecular machinery of programmed cell death as one of the essential disease states. Hepatitis is one of the cell death-associated diseases (33). Recently, the molecular machinery of fulminant hepatitis was suggested. Soluble Fas ligand produced from activated lymphocytes (34) stimulated Fas expressed on the cell surface of hepatocytes (4, 33, 35), and then stimulated Fas transduced an intracellular death signaling to activate caspase 3 (30, 36). In contrast, an essential role of CTL-induced cell death has been suggested in viral hepatitis (37). Two distinct pathways have been known as the molecular machinery for CTL-induced cell death: One is Fas ligand/Fas system, and another is Perforin/Granzyme B system.

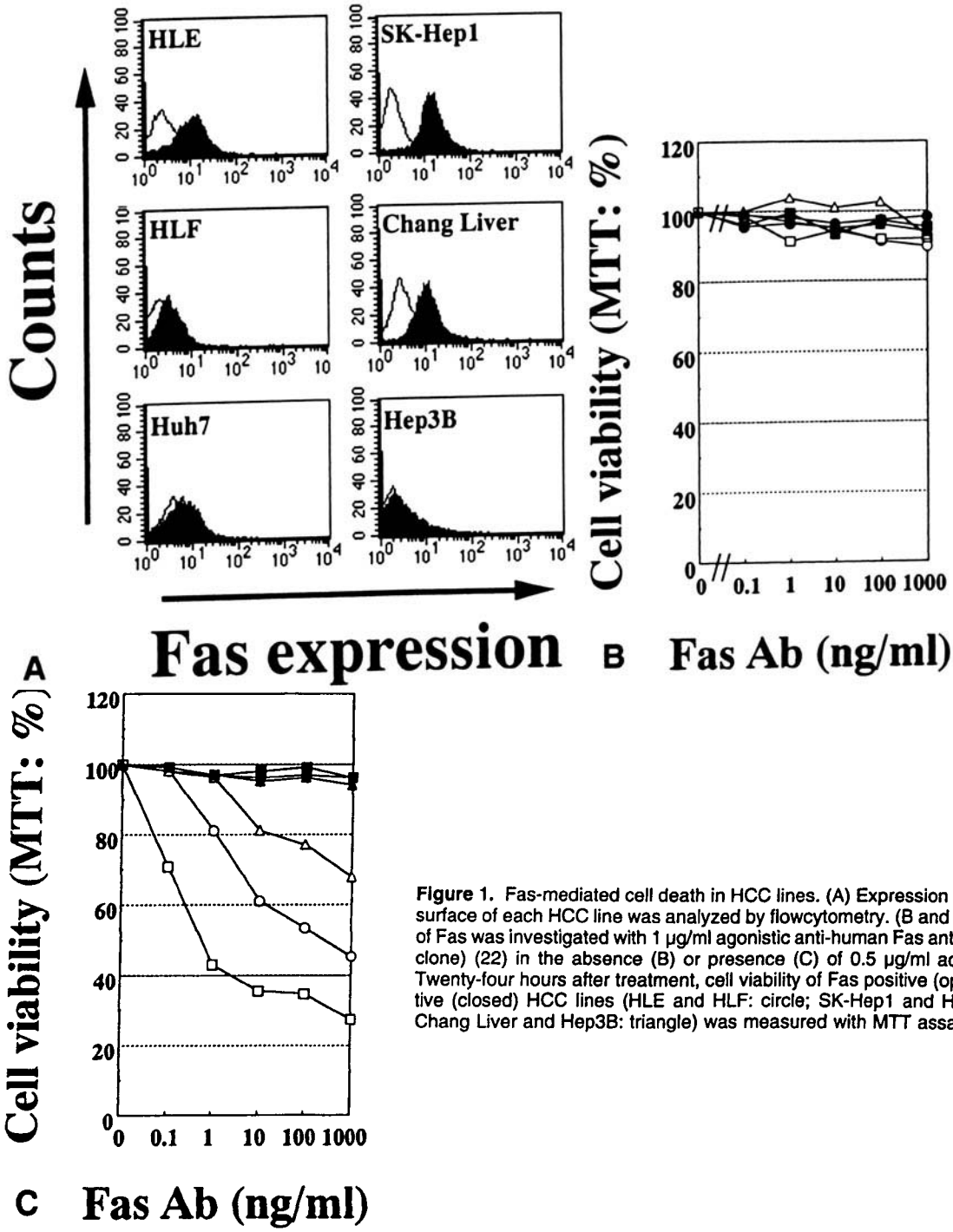


Figure 1. Fas-mediated cell death in HCC lines. (A) Expression of Fas on the surface of each HCC line was analyzed by flowcytometry. (B and C) Sensitivity of Fas was investigated with 1 μg/ml agonistic anti-human Fas antibody (CH-11 clone) (22) in the absence (B) or presence (C) of 0.5 μg/ml actinomycin D. Twenty-four hours after treatment, cell viability of Fas positive (open) or negative (closed) HCC lines (HLE and HLF: circle; SK-Hep1 and Huh7: square; Chang Liver and Hep3B: triangle) was measured with MTT assay.

Fas ligand/Fas system forms Fas-DISC (Fas-death inducing signaling complex: Fas-death domain/FADD/caspase 8) (16, 17, 38, 39), and then cytoplasmic serine proteinase and caspase 8 induce the activation of caspase 3 (15, 18). On the other hand, Granzyme B, which is secreted from CTL directly, activates caspase 3 (19) through Perforin-induced cell membrane pore (21). Thus, both systems act for the caspase 3 activation, whereas the domination in CTL-associated hepatitis has not been elucidated yet.

In the current study we approached the molecular machinery of CTL-induced cell death using HCC lines. Six HCC lines (HLE, HLF, Huh7, SK-Hep1, Chang Liver, and Hep3B) were used. Among tested HCC lines, Fas expression was detected in HLE, SK-Hep1, and Chang Liver cells. In addition, these three cell lines showed the Fas-mediated cell death only in the presence of actinomycin D, the same as human hepato blastoma HepG2 cells (12). Resistance to the Fas-mediated cell death in HepG2 cells is due to caspase 3 inactivation by cell cycle regulator $p21^{WAF1}$ and IAP family member ILP (12–14). Resistance to the Fas-mediated cell death observed in Fas-positive HCC lines may be due to the same system as HepG2 cells. All tested HCC lines

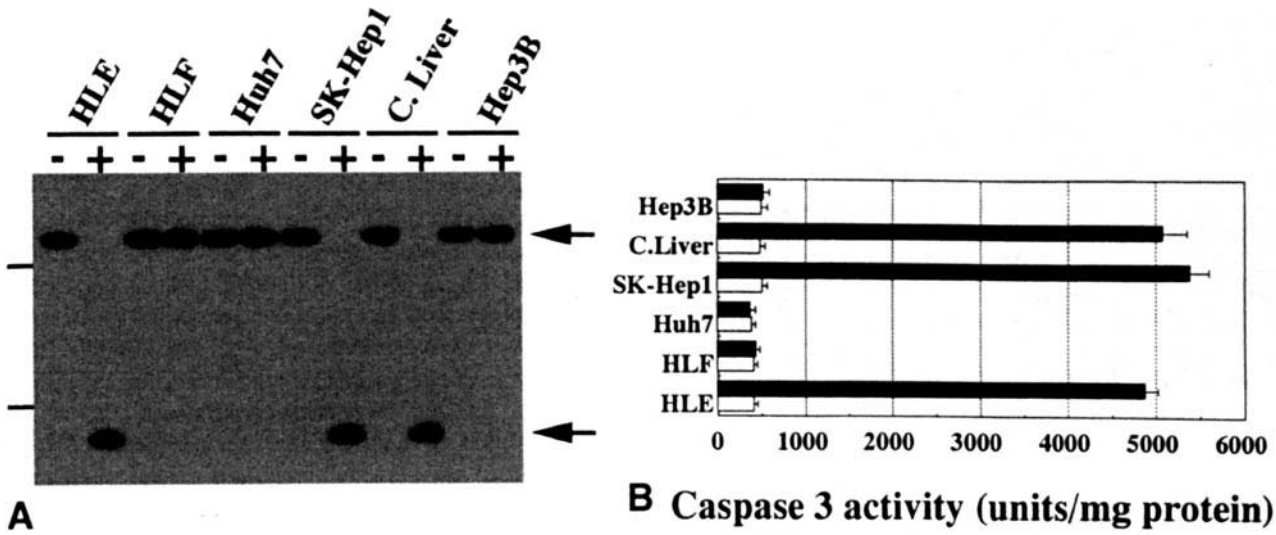
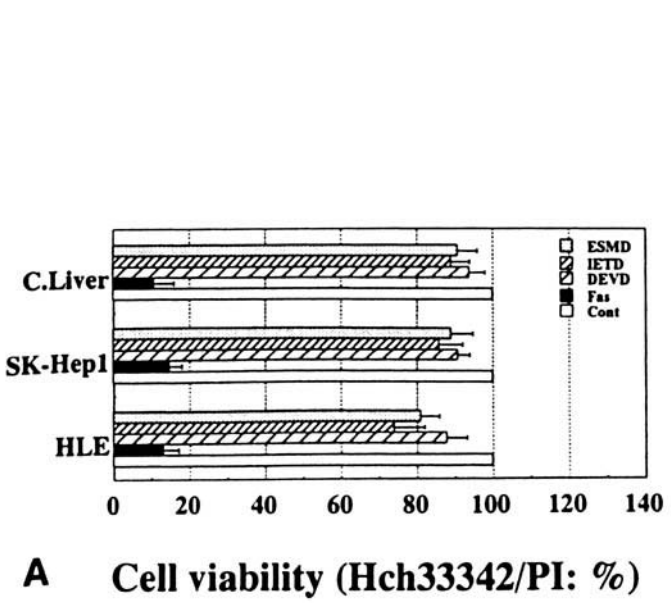
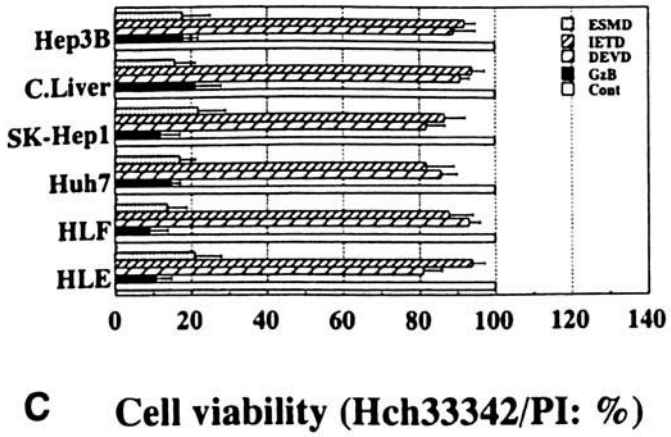


Figure 2. Caspase 3 in HCC lines. (A) The molecular shift and (B) proteolytic activity of caspase 3 in HCC lines treated with 1 μg/ml CH-11 clone in the absence (– or open) or presence (+ or closed) of 0.5 μg/ml actinomycin D for 16 hr were examined. The molecular shift of caspase 3 was observed by immunoblotting with monoclonal antibody for human caspase 3. Arrows on right show positions of immunostained bands; upper is procaspase 3 and lower is its active form. Bars on left show molecular weight markers, 30 and 20.1 kDa. Proteolytic activity of caspase 3 was measured with DEVD-MCA. Bars are SE from five independent experiments.





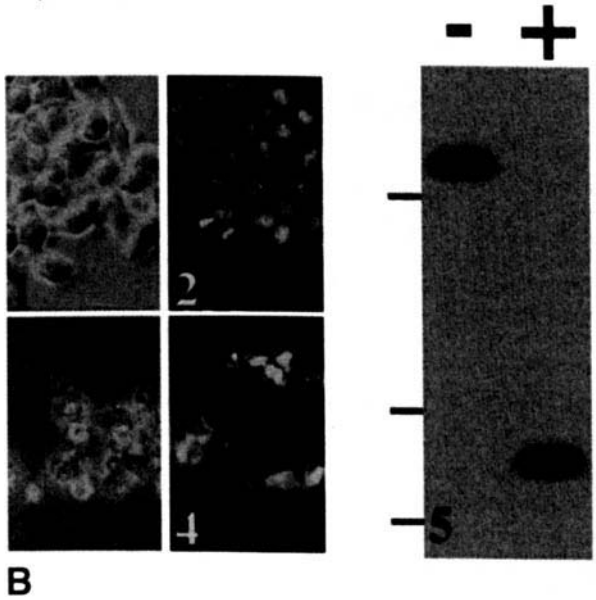


Figure 3. Characterization of Fas- and GzB-induced cell death. (A) Fas-initiated cell death signaling in Fas-positive HCC lines was investigated with three tetrapeptide inhibitors. Each of the cells pretreated with or without (Cont and Fas) each 100 μ*M* tetrapeptide inhibitor for 2 hr were incubated with (Fas) or without (Cont) 1 μg/ml CH-11 clone for 24 hr. After treatment, cells were stained with Hoechst 33342/PI (Hch33342/PI), and cell viability was measured as the ratio of intact nuclei to total nuclei (about 5000). Bars are SE from five independent experiments. (B) The trend in HLE cells was shown as the typical case of GzB-induced apoptotic cell death fashion and caspase 3 activation in HCC lines. Cells were microinjected with PBS (1, 2, and -) or PBS-diluted GzB (3, 4, and +), and then cell morphology (1 and 3; phase contrast), nuclear morphology stained with Hoechst 33342/PI (2 and 4; fluorescence microscopy), and caspase 3 molecular shift (5) were investigated after 24 hr. Bars on left of immunoblotting show positions of molecular weight markers, 30, 20.1, and 14.4 from top to bottom.(C) All HCC lines pretreated with or without (GzB) each tetrapeptide inhibitor for 2 hr were microinjected with PBS (Cont) or GzB. Twenty-four hours after microinjection, cells were stained with Hoechst 33342/PI (Hch33342/PI), and cell viability was measured as the ratio of intact nuclei in total nuclei (about 5000).

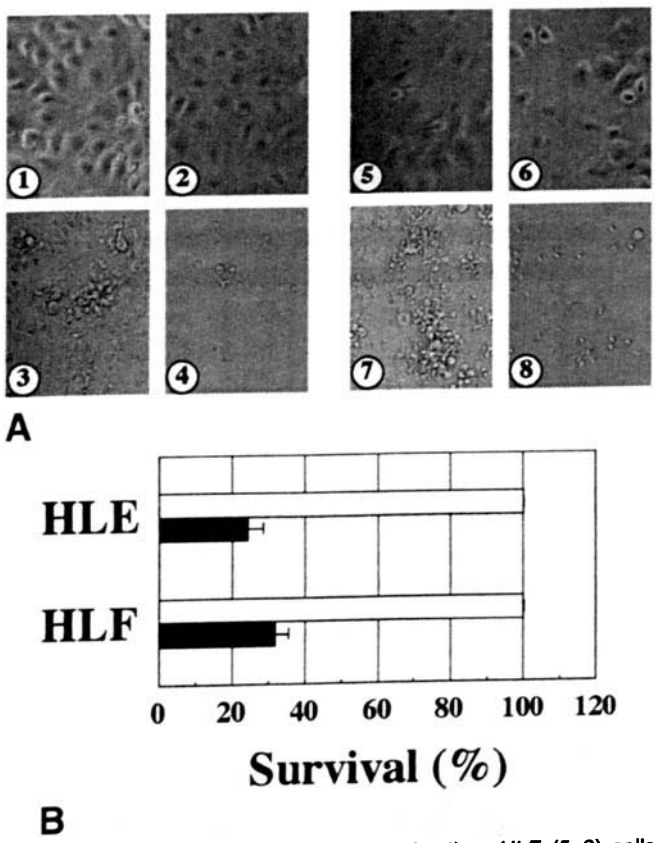


Figure 4. CTL-induced cell death. HLE (1–4) or HLF (5–8) cells were treated with CTL. Each cell line was treated with (3,4,7,8 in A: closed in B) or without (1,2,5,6 in A: open in B) CTL at E/T ratio = 2.5 for 4 hr. After treatment, observation of cell morphology (A) before (1,3,5,7) and after (2,4,6,8) PBS-wash, and measurement of survival rate (B) were performed.

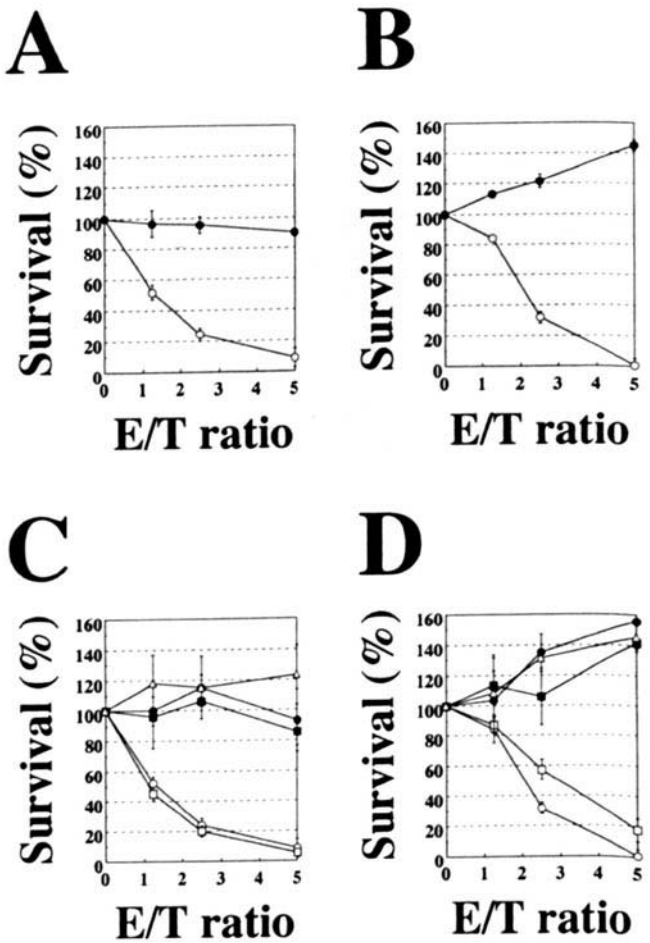


Figure 5. MHC class I restriction of CTLs and effects of proteinase inhibitor to CTL-induced cell death. (A) HLE or (B) HLF cells were pretreated with or without (○), anti-MHC class I monoclonal antibody (●) at 15 ug/ml for 2 hr. (C) HLE or (D) HLF cells were pretreated with or without (○) ac-IETD-CHO (●), ac-DEVD-CHO (■), Ac-ESMD-CHO (□) at 100 uM for 2 hr or CMA (△) at 100 nM for 2 hr, and then CTLs were reacted with each target cell line at indicated E/T ratio for 4 hr.

showed an expression of caspase 3, and Fas-positive HCC lines showed the molecular shift to active form upon the stimulation of Fas.

We demonstrated that Fas-positive HCC lines showed the caspase 3 activation during the Fas-mediated cell death. The molecular machinery of cell death signaling initiated by Fas ligand/Fas system is well documented (2); therefore, we also investigated the Fas-initiated cell death signaling in HCC lines using tetrapeptide inhibitors for caspase and its associated factor. Fas-mediated cell death in Fas-positive HCC lines was suppressed by all tested tetrapeptide inhibitors (Ac-DEVD-CHO for caspase 3) (10, 11); Ac-IETD-CHO for caspase 8 (18); and Ac-ESMD-CHO for caspase 3-activating serine proteinase (13, 15). Upon stimulation of Fas by Fas ligand, Fas-DISC (Fas-death inducing signaling complex) formation is initiated at first (39). Caspase 8 is one of the components of Fas-DISC (16,17) and its activated form proteolyzes caspase 3 at the p17 site after the caspase 3 proteolysis by serine proteinase at the p3 site (15). And, the activated caspase 3 proteolyzes poly (ADP-ribose) polymerase (PARP) (40), ICAD (41), and Acinus (42). On the basis of our current results, we also suggest that the Fas-mediated cell death signaling in HCC lines is also initiated by molecular machinery, the same as other cells, namely the sequential activation of serine proteinase, caspase 8, and caspase 3 after Fas-DISC formation.

The cell death induction machinery by CTL is also well documented, and two distinct processes are reported (21). One is the Fas ligand/Fas system. Fas ligand expressing on the cell surface of CTL stimulates Fas as a result of CTL-target cell contact, and the molecular machinery (same as above) is operated to induce cell death (21). Another is the Perforin/Granzyme B system. After CTL-target cell contact, Perforin is secreted from CTL and forms a pore on the target cell membrane, and then Granzyme B invades the target cell through Perforin-formed pores to activate caspase 3 (19, 21). In addition, perforin-independent GzB entry into target cells has been reported recently (43, 44). Thus, both distinct systems are operated to activate caspase 3 in the target cell; however, which system acts dominantly during CTL-induced cell death has not been clarified. The enzyme activity of Granzyme B is also suppressed by Ac-IETD-CHO (18). Therefore, we investigated the effects of tetrapeptide inhibitors during CTL-induced cell death. When CTLs were

reacted with target cells (Fas-positive: HLE; Fas-negative: HLF), cell death was induced independently on Fas expression. In addition, CTL-induced cell death was suppressed by AC-DEVD-CHO or Ac-IETD-CHO, but not by Ac-ESMD-CHO. The prevention of CTL-induced cell death by Ac-DEVD-CHO is due to caspase 3 inactivation, and we suggest that the target molecule for CTL-induced cell death prevention is caused by Granzyme B inactivation, rather than by caspase 8 inactivation, since Ac-ESMD-CHO, which is an essential suppresser for Fas-mediated cell death, did not suppress, and Fas expression was not involved with CTL-induced cell death. In addition, the inhibitory effect of CMA on CTL-induced cell death was observed in the current study. However, antiapoptotic protein *Bcl*-2 (45–47), which suppresses the Fas-mediated cell death (48), also did not show any effects in CTL-induced cell death (Midori Hayashida, unpublished data). These results suggest that CTL-induced cell death in HCC lines is dominantly initiated by the Perforin/Granzyme B system.

Cell death induction by CTLs is an important thesis for understanding tumor immunotherapy. Two distinct processes, Fas Ligand/Fas and Perforin/Granzyme B systems, have been well documented; however, our current results indicated that cell death induction by CTL in HCC lines was initiated dominantly by Perforin/Granzyme B system. It has also been well documented that TNF-α plays an essential role for hepatocytic cell death during lipopolysuccharide (LPS) shock (49); therefore, the TNF-α/TNF-RI system may be involved in HCC cell death. TNF-α/TNF-RI system-transduced cell death is also mediated by Fas-activated serine proteinase (caspase 3 activator, A. Suzuki, unpublished data); however, its tetrapeptide inhibitor did not suppress CTL-induced cell death. Here, we propose that the Perforin/Granzyme B system is the dominant inducer for CTL-induced cell death when CTLs contact with target tumor cells.

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