The Dominant Role of CPP32 Subfamily in Fas-Mediated Hepatitis (44256)

ATSUSHI SUZUKI[1]

Drug Safety Research Laboratory, Daiichi Pharmaceutical Co., LTD, Tokyo R&D Center 16-13, Kitakasai-1, Edogawa-ku, Tokyo 134, Japan

Abstract. **Fas is a cell surface molecule that transduces the apoptotic death signaling on the stimulation of Fas ligand, and plays the dominant role in various disease states. The lethal effect of Fas antibody in mice has been reported, and this experimental procedure has been used as the model for hepatitis. Recently, the prevention of this Fas antibody-induced hepatitis by the broad caspase inhibitor (z-VAD.fmk) has been reported. In the present study, we additionally demonstrated that the CPP32 subfamily, rather than the ICE subfamily, plays the dominant role in the Fas antibody-induced hepatitis. Fas antibody-injection induced chromosomal DNA fragmentation and CPP32 subfamily-activation in both the liver and lung. Tissue damage observed in the lung was weak as compared with liver damage. When mice were exposed to DEVD-CHO (specific inhibitor of CPP32 subfamily), this lethal effect of Fas antibody, tissue destruction, and CPP32 subfamily-activation were prevented. In contrast, YVAD-CHO (specific inhibitor of ICE subfamily) could not prevent the lethal effect of Fas antibody. We propose here that the CPP32 subfamily plays the dominant role in Fas-mediated hepatitis, and DEVD-CHO would be an effective cure for hepatitis.**

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Cell surface molecule Fas, a type-I transmembrane protein belonging to the nerve growth factor/tumor necrosis factor receptor family, transduces the apoptotic death signaling on stimulation with Fas ligand or anti-Fas antibody (Fas Ab) (1–3). Fas plays the dominant role in physiological cell death, and its endogenous expression is encountered in liver, lung, thymus, heart, ovary, and vagina (4, 5). Recently, the lethal effect of Fas Ab in mice was reported, in which it was shown that this lethal effect was due to fulminant liver destruction (6). Various investigations into the molecular machinery of various types of hepatitis have also demonstrated the direct involvement of Fas ligand/Fas system (7–9), and Fas Ab injection into mice was established as the model for hepatitis (10, 11).

CED-3 death gene, identified from *Caenorhabditis elegans,* shows high similarity to interleukin-1β converting enzyme (ICE) (12, 13). Recently, various ICE/CED-3 homologs that were recently termed "caspase (14)" have been identified, and an important role in apoptotic cell death has been reported. Especially, CPP32 subfamily (15) plays the dominant role in apoptotic death signaling (16–19). On the basis of the machinery by which they induce apoptotic death signaling, two types of CPP32 subfamily are known: one is CPP32/Yama/Apopain (caspase 3), which does not physically interact with the cytoplasmic region of Fas; and another is FLICE/MACH (caspase 8), which can directly interact with the death domain of Fas (18, 19). In Fas-mediated apoptosis, caspase 3 is activated by ICE subfamily-activation (ICE cascade; Ref. (16)). Thus, the CPP32 subfamily plays the dominant role in the downstream of Fas-initiated death signaling.

Bcl-2 oncoprotein was originally identified through study of the t(14; 18) translocation present in human B-cell follicular lymphomas (20), and is unique in that it inhibits apoptosis rather than promoting cell proliferation (21, 22). Bcl-2 prevents Fas-mediated apoptosis *in vitro* (23). Because Bcl-2 expression is not encountered in liver (24), the effect of Bcl-2 in Fas Ab-induced fulminant liver destruction was investigated in a Bcl-2 transgenic mouse. Overexpression of Bcl-2 in mouse liver showed the resistance to Fas Ab-induced fulminant liver destruction (10, 11). It is thought that Bcl-2 prevents the upstream portion of the ICE cascade (25). Therefore, this finding in Bcl-2 transgenic

[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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mice suggests that the prevention of Fas-initiated death signaling would be an effective cure for hepatitis. In addition, the prevention of Fas Ab-induced hepatitis by the systemic injection of broad caspase inhibitor to mice has recently been reported (26). In the present study, therefore, we examined the protective effect of the CPP32 subfamily specific peptide-inhibitor DEVD-CHO in Fas Ab-induced lethal effect.

Materials and Methods

Fas Ab- or DEVD-CHO-Injection. Male Slc/ddy, 5-week-old mice were used for experiments. Sixteen mice were used in each group. Anti-mouse Fas antibody (Jo2 clone and Ref. 6) was purchased from Pharmingen (San Diego, CA) and diluted with PBS. Ten micrograms of Fas Ab were injected intravenously, with an equal amount of hamster IgG diluted with PBS injected as control. After injection, lung, heart, liver, and thymus were removed, and used for chromosomal DNA analysis, histological analysis, and the measurement of CPP32 subfamily-activity. CPP32 subfamily-inhibitor DEVD-CHO was purchased from Peptide Laboratories (Osaka, Japan) and dissolved with DMSO. The DMSO-dissolved DEVD-CHO was diluted from PBS (final conc. of DMSO is 1%), and 0.1 or 0.5 mg of DEVD-CHO were injected intravenously. Each experiment was performed three times.

Chromosomal DNA Analysis. To examine whether Fas Ab injection induces apoptosis in each tissue, chromosomal DNA fragmentation, one of apoptotic fashion (27), was analyzed as previously described (5, 28). Tissues were digested with lysis buffer 100 m*M* NaCl, 25 m*M* ethylenediaminetetra-acetic acid (EDTA) (Wako), 100 m*M* Tris-HCl (pH 8.0), 0.5% SDS, and 0.3 mg/ml proteinase K (Wako) at 50°C for 14 hr after homogenization. Samples were extracted three times with phenol/chloroform/isoamyl alcohol (PCIA; 25:24:1) and then once with chloroform. An equal volume of isopropanol was added to the aqueous phase, and then DNA was precipitated for 1 hr at −20°C. The DNA was dissolved in 10 m*M* Tris 50 m*M* EDTA (pH 8.0), and then treated with 50 μg RNase (Wako) for 1 hr at 37°C. After final PCIA extraction, DNA was precipitated as described above. Purified DNA was measured spectrophotometrically at A260/A280, and the same amount of DNA (1 μg/lane) was electrophoretically separated in 2% agarose gel in TBE (45 m*M* Tris/45 m*M* bolic acid/1 m*M* EDTA) for 1 hr at 100 volts/hr. Gels were stained with ethidium bromide (0.5 μg/ml) for 5 min and rinsed with distilled water.

Histological Analysis. Tissues from mice were fixed in formalin, embedded in paraffin, and serially sectioned at 8 μm. The sections were stained with Delafield's hematoxylin and eosin (HE) or Hoechst 33342. The Hoechst 33342 staining procedure was performed as previously described (17) with some modifications. Sections were treated with xylene and EtOH, and then washed with PBS. After washing, sections were reacted with Hoechst 33342 (1 μ*M*) for 18 hr.

Protein Extraction and Measurement of CPP32 Subfamily-Activity. Protein extraction and the enzyme assay were performed as previously described (17) with some modifications. Tissues were removed, washed with PBS, and minced in PBS containing 1 m*M* EDTA. After the addition of 10 μ*M* (final conc.) digitonin (Sigma Chemical Co., St. Louis, MO), tissues were incubated at 37°C for 30 min. Lysates were collected by centrifugation (15000 rpm/5 min), and protein concentration was measured using a DC protein assay kit (Bio Rad, Hercules, CA).

For assay of CPP32 subfamily-activity, aliquots were incubated with 10 μl of DEVD-MCA (Peptide Lab.; 50 μ*M*), and the release of amino-4-methylcoumarin was monitored with a spectrofluorometer.

Results and Discussion

When mice (Slc/ddy; male, 5 weeks old) were injected intravenously with 10 μg of anti-mouse Fas antibody (Fas Ab; Jo2 clone and Ref. 6), all Fas Ab-injected mice died within 6 hr (Fig. 1). To examine tissue damage, chromosomal DNA analysis and histological study were performed in Fas-expressing tissues, namely lung, heart, liver, and thymus (4). In control mice (hamster IgG-injected mice and Ref. 6), tissue damage was not encountered in all tissues (Fig. 2). In contrast, tissue damage detected by chromosomal DNA fragmentation was detected in lung, liver, and thymus of Fas Ab-injected mice, but not in heart (Fig. 2a). It has been reported that intraperitoneal injection of Fas Ab induces thymocyte apoptosis, and that $CD4^+/CD8^+$ thymocytes die as the target of Fas Ab (29). We suggest that the apoptosis encountered in thymus of Fas Ab-injected mice is due to $CD4^+/CD8^+$ thymocyte apoptosis. HE- and Hoechst 33342-staining analysis revealed that cells in the liver and

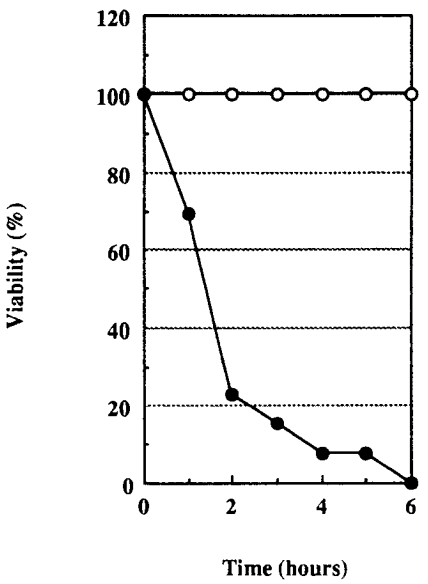

Figure 1. Lethal effect of Fas Ab in mice. 100 μl of 1% DMSO in PBS (DMSO-PBS) was injected intravenously, followed by intravenous injection of Fas Ab 10 μg (closed circle) or hamster IgG (open circle).

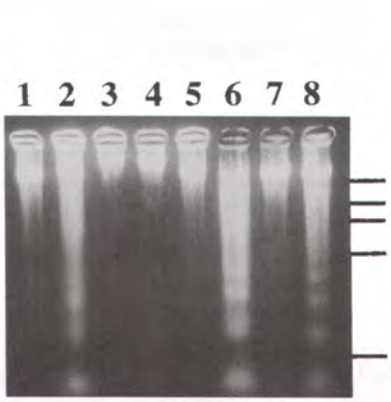

Figure 2. Tissue damage induced by Fas Ab-injection. (a) Chromosomal DNA fragmentation analysis. Chromosomal DNAs were extracted from lung (Lanes 1 and 2), heart (Lanes 3 and 4), liver (Lanes 5 and 6) or thymus (Lanes 7 and 8) of mice injected with hamster IgG (Lanes 1, 3, 5, and 7) or Fas Ab (Lanes 2, 4, 6, and 8). Chromosomal DNAs extracted from tissues of Fas Ab-injected mice that died at 2 hr after injection were indicated. Chromosomal DNAs were separated on 2% agarose gels and stained with EtBr. Bars on the right of the photograph show the position of DNA marker 1357, 1078, 872, 603 and 194 bp (from top to bottom). (b) Histological analysis of liver and lung damage. Liver (upper panels: 1–4) and lung (lower panels: 5–8) were removed from intact mice and stained with HE (1 and 5; 400×). Liver and lung of Fas Ab-injected mice that died at 2 hr after injection were also removed and stained with HE (2 and 6; 400×) or Hoechst 33342 (3, 4, 7, and 8; 3 and 7: 400×; 4 and 8: 1000×). Arrowheads (black and yellow in each panel) show the typical apoptotic cells showing nuclear condensation and/or fragmentation.

lung showed some typical apoptotic fashion (27), such as nuclear fragmentation and condensation (Fig. 2b), whereas lung damage was very weak as compared with liver damage. We suggest that the lethal effect of Fas Ab is dominantly due to fulminant liver destruction, and that light lung damage may cause some effect to the lethal effect of Fas Ab.

Hasegawa *et al.* reported the dominant role of the CPP32 subfamily in Fas-mediated apoptosis (17). In addition, if the ICE cascade operates in Fas-mediated hepatitis, the CPP32 subfamily plays the dominant role at the down stream portion (16, 30, 31). Therefore, the direct involvement of the CPP32 subfamily in Fas Ab-induced tissue damage was examined. Because the CPP32 subfamily cleaves poly (ADP-ribose) polymerase (PARP) (15, 32), MCA-modified tetrapeptide (DEVD-MCA) coding the

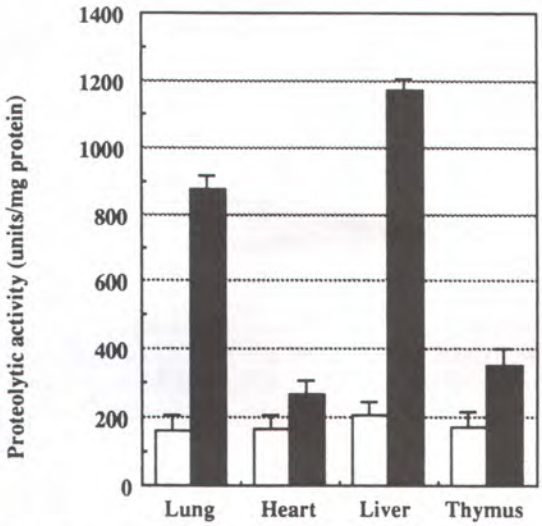

Figure 3. Proteolytic activity of CPP32 subfamily. Proteins were extracted from tissues from mice injected with hamster IgG (open column) or Fas Ab (closed column).

cleavage site of PARP can be used as the substrate (17). When mice were exposed to Fas Ab, a drastic elevation of CPP32 subfamily-activity was encountered in lung and liver, but not in heart and thymus (Fig. 3).

Various investigations have reported that CPP32 subfamily-activation initiated by Fas is prevented by the synthesized tetrapeptide inhibitor DEVD-CHO *in vitro* (17, 32, 33). Therefore, we examined the effect of DEVD-CHO on the lethal effect of Fas Ab. Mice were intravenously preinjected with DEVD-CHO, and then exposed to Fas Ab. Even single injection of DEVD-CHO rescued 18.75% (0.1 mg/mouse), 50% (0.5 mg/mouse) 75% (1 mg/mouse) or 100% (5 mg/mouse) of the mice from the lethal effect of Fas Ab (Fig. 4a), and fulminant liver destruction was also prevented (Fig. 4b). Broad caspase inhibitor z-VAD.fmk also rescued 100% of the mice from the lethal effect of Fas Ab by the 1-hr interval injection after Fas Ab-injection (26). In the present study, single injection of DEVD-CHO (5 mg/mouse) was effective (100% rescue) even 30 min after Fas Ab-injection (data not shown). The effect of a single injection of ICE inhibitor (YVAD-CHO) on the lethal effect of Fas Ab was also examined. Whereas, YVAD-CHO injection (0.5 mg/mouse) could rescue only 33.3% of the mice from the lethal effect of Fas Ab, and 15 mg/mouse were required to rescue 100% of the mice from the lethal effect of Fas Ab (data not shown). Therefore, we propose here that DEVD-CHO is more effective than other caspase inhibitors. In addition, DEVD-CHO exposure to mice completely prevented Fas Ab-induced CPP32 subfamily-activation (Fig. 4c).

The present study demonstrated that Fas Ab-injection in mice induced liver and lung damage mediated by CPP32 subfamily activation. In general, an abnormality of the lungs in patients carrying hepatitis has been known. Although, the dominant role of the Fas ligand/Fas system in hepatitis has already been demonstrated, there is no report that suggests the possible involvement of the Fas ligand/Fas system in lung damage. The lethal effect of Fas Ab was prevented by the pre- and postinjection of DEVD-CHO. The administration of DEVD-CHO may be effective in the treatment of hepatitis. Various disease states triggered by caspase activation have been reported (3, 34). In addition, chemotherapeutic agents also induced apoptosis accompanied by caspase activation (31, 35, 36) and showed various side effects. The current results additionally suggest that DEVD-CHO may become the basic model of the new drug that is

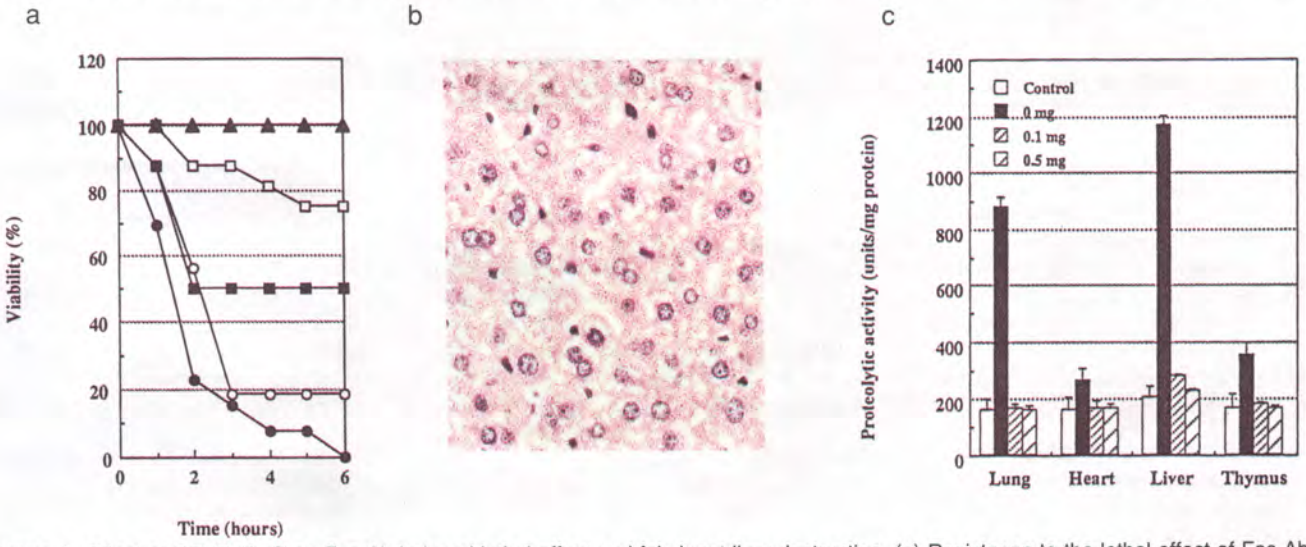

Figure 4. Effect of DEVD-CHO on Fas Ab-induced lethal effect and fulminant liver destruction. (a) Resistance to the lethal effect of Fas Ab in mice. Mice were injected intravenously with 100 µl of DMSO-PBS (closed circle), or 0.1 (open circle), 0.5 (closed square), 1 (open square) or 5 (closed triangle) mg of DEVD-CHO. After 2 hr, mice were injected intravenously with 10 µg of Fas Ab. (b) Histological analysis of liver from DEVD-CHO pretreated mice. Liver was removed from both DEVD-CHO (0.5 mg) and Fas Ab-treated mice that were alive at 6 hr after Fas-Ab injection and stained with HE (400×). As compared with intact mice liver (Fig. 2b-1), there was no significant difference. (c) Effect of DEVD-CHO on proteolytic activity of CPP32 subfamily. Proteins were extracted from tissues of rescued mice. Mice injected with DMSO-PBS (0 mg), or 0.1 (0.1 mg) or 0.5 (0.5 mg) mg of DEVD-CHO. After 2 hr, mice were injected intravenously with 10 µg of Fas Ab.

an effective cure of cell death–associated diseases and side effects.

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