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

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Summary

Mice injected with anti-Fas antibody die within a few hours with total liver destruction due to massive apoptosis of hepatocytes. We show that this is preceded and accompanied by the sequential activation of cysteine proteases of the interleukin 1β–converting enzyme (ICE) and CPP32 types in the cytosol of the hepatocytes, and that proCPP32 cleavage and enzymatic activity can be prevented by intravenous injections of the tripeptide *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk), an inhibitor of ICE-like proteases. Four Z-VAD.fmk injections at 1-hour intervals abolished all signs of liver damage after anti-Fas antibody injection and resulted in 100% long-range recovery, without residual tissue damage, from a condition otherwise uniformly fatal within <3 hours. This treatment was effective even when delayed until some liver DNA degradation was already detectable. Injections of the tetrapeptide Ac-YVAD.cmk, more specific for the ICE-like subfamily of cysteine proteases but less cell permeable, also gave protection, but at higher doses and when injections started before that of anti-Fas antibody. These observations afford a way of temporarily modulating a number of apoptotic processes in vivo and may have important therapeutic implications in some human diseases.

There is now conclusive evidence that the process of apoptosis or programmed cell death (PCD) results from the activation of members of a new family of cysteine proteases with a specificity of cleavage for aspartate in the P1 position. The decisive importance of this mechanism as an effector of PCD was revealed by the discovery that the *ced-3* gene of *Caenorhabditis elegans*, which is required for cell death occurring during the normal development of this nematode (1), encodes for a protein related to the mammalian IL-1β–converting enzyme (ICE) (2), an aspartate-specific cysteine protease, and that, in certain conditions, overexpression of ICE itself in mammalian cells can lead to apoptosis (3). Several other members of this protease family have now been identified and are presently subdivided into three subfamilies, each containing variants: the ICE, CPP32 (also called YAMA, apopain, or prICE), and Ich-1 (or Nedd-2) subfamilies; all these enzymes are synthesized as inactive proenzymes requiring cleavage at specific Asp residues to be transformed into active proteases: thus, at least some of these proteases can activate each other in the form of a proteolytic cascade and/or may undergo, once activated, autoprocessing, allowing the potentially lethal amplification of a minor initial proteolytic process (for review, see reference 4). Short peptides corresponding to the cleavage site of some of these cysteine proteases have been used as inhibitors, with the Tyr-Val-Ala-Asp (YVAD) and the Asp-Glu-Val-Asp (DEVD) motifs being rather specific inhibitors of the ICE and CPP32 subfamilies, respectively (5). Provided their extracellular concentration is high enough, addition of these or related inhibitory peptides to cell cultures prevents many forms, but not all, of PCD (6–8).

One of the best studied ICE-related protease cascades involved in apoptotic cell death is that induced by the triggering of the Fas (APO-1, CD95) receptor on Fas-bearing cells in vitro (6, 7). It has been shown that ICE-like protease(s) and CPP32 are activated in sequence after Fas stimulation (9), and that the first proteolytic event may result from the activation of a novel cysteine protease, which is bound to the FADD or MORT protein (itself bound to the intracellular domain of the Fas receptor) and has a Ced-3–like structure (10, 11). The accumulation of activated CPP32-like proteases thus appears to represent a downstream event in this proteolytic cascade. The most dramatic effect of Fas triggering in vivo as the result of a single injec-

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tion of an agonistic anti-Fas antibody into mice is the massive apoptotic death of hepatocytes, which results in a few hours in total liver destruction and death (12). In this report we show that this event, which is also accompanied by the sequential activation of ICE- and CPP32-like proteases in the cytoplasm of the hepatocytes, can be completely and permanently prevented by the concomitant injections of high doses of the tripeptide benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk) (13) and a more specific inhibitor of ICE-like proteases, acetyl-Tyr-Val-Ala-Asp chloromethylketone (Ac-YVAD.cmk).

Materials and Methods

Mice. Mice used in this study were 3–4-wk-old (16–18 g) C57BL/6 females bred in the animal facility of the University Medical Center of Geneva.

Anti-Fas Antibody and ICE-like Inhibitor Injections and Histologic Examination. Mice were injected intravenously with 10 μg of a purified hamster mAb (Jo2) against mouse Fas antigen (12) diluted in 80 μl of 0.9 g/liter NaCl solution. Z-VAD.fmk (Enzyme Systems Products, Dublin, CA), Z-D.cmk, and Ac-YVAD.cmk peptides (BACHEM Feinchemikalien AG, CH), were dissolved in DMSO at a concentration of 100 mg/ml. All peptide injections were intravenous and started 5 min after anti-Fas antibody injection, except when indicated otherwise. Mice were killed at various times or autopsied immediately after death, and fragments of tissues were fixed in vivo with 4% paraformaldehyde in PBS, embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin.

Western Blot Analysis. Minced liver fragments were homogenized at 4°C with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 2 μg/μl aprotinin and 1 mM PMSF. After centrifugation at 15,000 *g*, 30 μg of supernatant protein was loaded per lane on 10% polyacrylamide gels and electrophoresed. After transfer to nitrocellulose membranes and exposure to 5% nonfat milk TBS-T (20 mM Tris HCl, 500 mM NaCl, pH 7.5, and Tween 0.1%), the washed membranes were incubated for 1 h at 20°C with a rabbit anti-murine ICE at a 1:3,000 dilution (kind gift of J. Tschopp, Institut de Biochimie, ISREC, Lausanne, Switzerland), a rabbit anti-ICE p10 at a dilution of 1:200 (M20; Santa Cruz Biotech Inc., CA), a rabbit anti-human CPP32 p17 at a dilution of 1:7,500 (kind gift of D. Nicholson, Merck Frosst, Pointe-Claire-Dorval, Quebec, Canada), or anti-LAP3 (kind gift of V.M. Dixit, University of Michigan, Ann Arbor, MI). Goat anti-rabbit IgG (Santa Cruz Biotech. Inc., Santa Cruz, CA) was used as second antibody at a concentration of 400–800 ng/ml. Membranes were washed with TBS-T, incubated in enhanced chemiluminescence detection reagents (Amersham International, Amersham, UK) at room temperature, and exposed to Hyperfilm-MP films (Amersham International).

Fluorometric Analyses. Liver protein extracts were prepared by Dounce homogenization of 20 mg of tissue in a hypotonic buffer (25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin and aprotinin). Homogenates were centrifuged at 15,000 rpm for 10 min and the supernatants were used. 20 μg of the extracted proteins was incubated with the fluorescent substrates Z-YVAD-AFC or Z-DEVD-AFC (Enzyme Systems Products) at a concentration of 25 mM in 50 mM Hepes, 1% sucrose, 0.1% CHAPS (3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate), and 5 mM dithiothreitol in a volume of 1 ml. The fluorescence of the cleaved substrates was determined using a spectrofluorometer set at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. One unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 25°C in 30 min.

DNA Fragmentation Detection. For the detection of oligonucleosomes, a cell death detection ELISA kit (Boehringer Mannheim AG, Rotkreuz, Switzerland) was used according to the manufacturer's instructions adapted for the use of tissue fragments. Briefly, small pieces of liver (25–40 mg) were weighed and homogenized in the provided lysis buffer, incubated for 30 min at room temperature, and after a 10-min centrifugation at 2,000 rpm, 20 μl of the supernatant was submitted to the ELISA test. For electrophoretic detection of DNA cleavage products, liver extract supernatants were precipitated overnight with isopropanol. The 15,000-rpm pellets were then digested overnight with proteinase K, extracted with phenol-chloroform-isoamylalcohol, treated with RNase A, precipitated with ethanol, and submitted to electrophoresis in 2% agarose gels.

Serum Aminotransferase Determination. Quantification of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) in the sera was made with a standard clinical automatic analyzer (model 7150; Hitachi).

Results

Intravenous injection of 10 μg of the Jo2 anti-Fas mAb was used throughout these experiments. This amount invariably killed all 15–18-g C57BL/6 mice within 3 h; when killed after 2 h, all mice already had a severely shrunken, massively hemorragic liver, with extensive lesions of hepatocyte apoptosis on histologic sections (Fig. 1 *b*, compared with normal liver, Fig. 1 *a*). Evidence of hepatocyte damage became clearly detectable only after 60 min; at this time, blood levels of liver aminotransferases were almost unchanged (Fig. 2 *a*), and in liver lysates DNA oligonucleosomes characteristic of apoptosis were only detectable by a sensitive immunologic assay (Fig. 2 *b*); after 60 min, the blood aminotransferases rapidly increased to very high levels until death (Fig. 2 *a*) and DNA fragmentation in the liver, as detected on agarose gels, became massive (Fig. 2 *b*). Proteolytic activity of the ICE and CPP32 type was as-

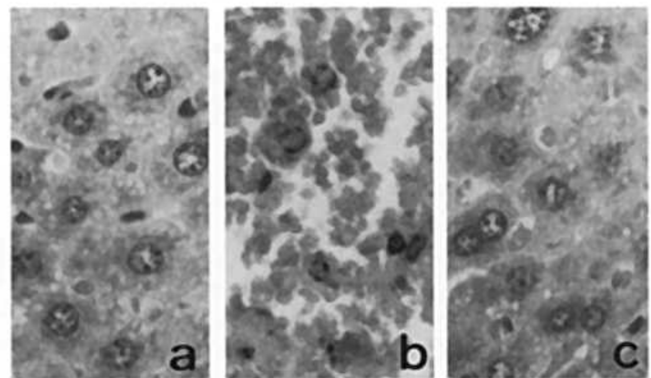

Figure 1. Liver histology, detail. (*a*) Normal mouse. (*b*) Mouse killed 2 h after injection of anti-Fas antibody. The architecture of the liver parenchyma is destroyed with hemorrhagic foci, several apoptotic hepatocytes are seen. (*c*) Mouse killed 2 h after antibody injection, followed after 5 min by injection of 500 μg Z-VAD.fmk. Original magnification ×400.

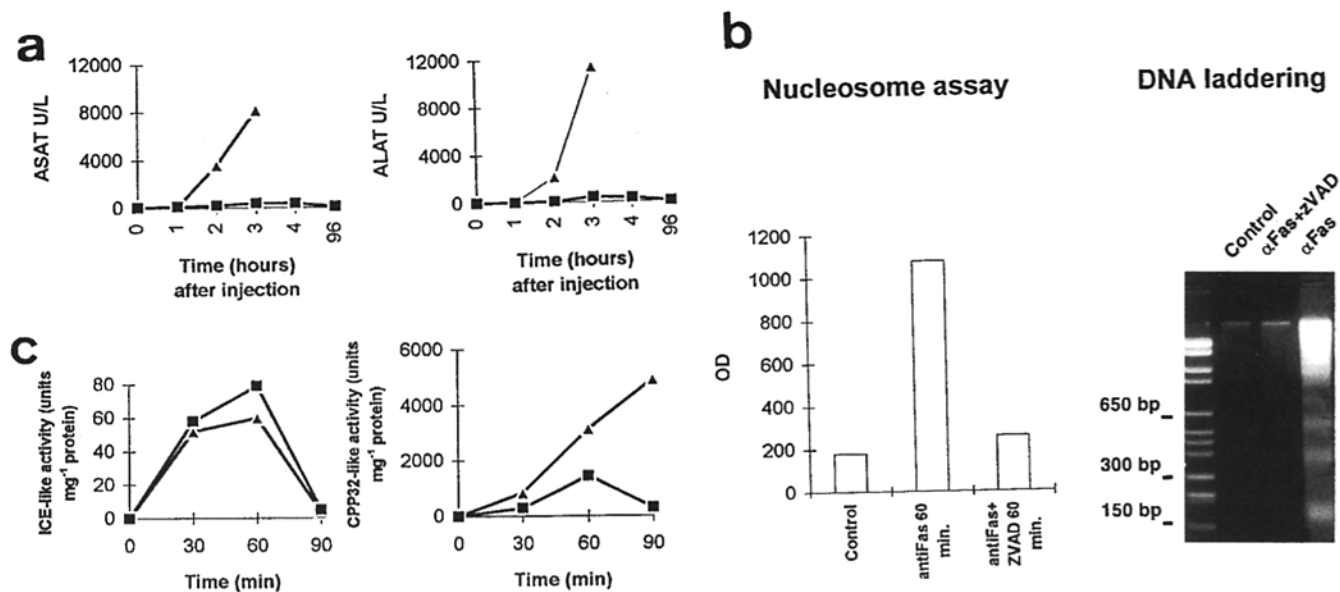

Figure 2. (*a*) Prevention by Z-VAD.fmk injections of the increase of ASAT (*left*) and ALAT (*right*) concentrations in the serum after Fas-induced liver damage. ▲, control mice. ■, treated mice. (*b*) Prevention of Fas-induced liver DNA fragmentation after Z-VAD.fmk injections. (*Left*) DNA fragmentation assay (*nucleosome*). Normal liver (*left column*); middle: liver 1 h after anti-Fas antibody injection (*middle column*), effect of Z-VAD.fmk injection 5 min after anti-Fas injection (*right column*). (*Right*) DNA laddering on agarose gel 2 h after anti-Fas injections, with or without Z-VAD.fmk injection. (*c*) Activation of ICE-like and CPP32-like proteases during Fas-induced liver damage in vivo, using cleavage assays of the fluorescent substrates Z-YVAD-AFC (for ICE-like proteases; *left*) and Z-DEVD-AFC (for CPP32-like proteases; *right*) in the presence of the same liver cytosolic extracts used for both assays. ▲, control. ■, Z-VAD–treated mice.

sessed in the liver lysates of mice killed at 30-min intervals after antibody injection, by using as substrates the Z-YVAD-AFC and Z-DEVD-AFC peptides, which correspond, respectively, to an ICE and a CPP32 cleavage site (5). ICE-like activity rapidly rose in liver lysates after 30 min but in a transient way, and returned to normal values at 90 min; in contrast, CPP32-like activity rose continuously until 120 min, at the time of very severe hepatocyte damage (Fig. 2 *c*). Cleavage of proICE and proCPP32 was also directly explored by Western blotting of the liver lysates (Fig. 3). A very limited cleavage of proICE, indicated by the appearance of a 36-kD band (14), was detectable after 60 min; a decrease in proCPP32 was observed after 60 min and corresponded to a limited cleavage of the proenzyme as shown by the detection of a 17-kD band of weak intensity. No LAP-3, a protease of the CPP32 family, was detectable, suggesting that this enzyme is not present in the liver (15). The nuclear poly(ADP-ribose) polymerase, a substrate for CPP32 whose cleavage is characteristic of apoptotic nuclei (16), could not be clearly visualized, perhaps because this protein is less abundant in the nuclei of the nondividing hepatocytes than in those of dividing cells, where it is usually observed.

Mice were then injected with anti-Fas antibody followed 5 min later by intravenous injections of various amounts of the Z-VAD.fmk peptide. In a pilot experiment, two mice injected with 1 mg or 0.25 mg (about 2 or 0.5 μM, respectively) of the peptide survived 15 and 7 h, respectively, but had severely damaged liver at autopsy, compara-

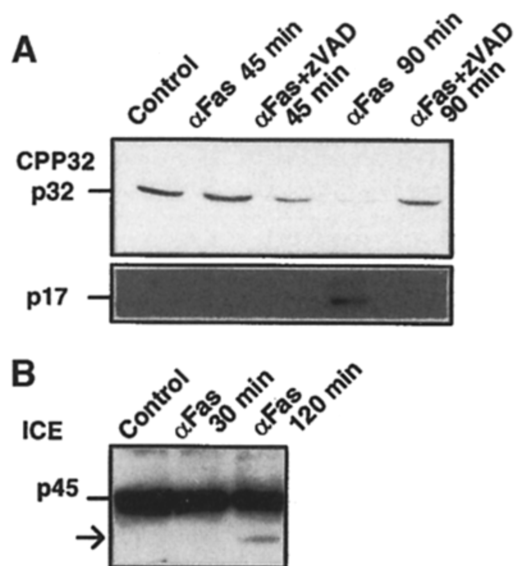

Figure 3. Western blots for the detection of CPP32 and ICE in liver cytosolic extracts. (*a*) Anti-CPP32 antibody. In addition to the p32 band of proCPP32, a band of p17 is present in the liver 90 min after antibody injection and is not detectable in the liver lysates from a Z-VAD.fmk-treated mouse. Exposure time of the film was 5 min for p45 and 15 min for p17. (*b*) Anti-ICE antibody: a faint band (p36, *arrow*), corresponding to the initial processing of proICE (p45) is distinguishable in liver lysates after anti-Fas antibody injection. Whereas in other experiments the faint p36 band was seen after 60 min, here it was more clearly seen in the 120-min liver lysate.

ble to that of control mice, which died within 3 h; three mice injected with 0.5 mg of the peptide were killed after 2 h and had macroscopically and histologically normal liver (Fig. 1 *c*). On this basis, it was decided to prolong exposure to the protecting peptide by using the following schedule: an initial injection of 0.25 mg, followed by three injections of 0.1 mg at 1-h intervals. Three experiments were then performed, corresponding to a total of 15 mice injected according to this schedule and 20 control mice: all control mice died within 3 h, whereas the 15 Z-VAD–injected mice survived and were in apparent good health days or weeks later. In a last experiment involving five mice and their controls, this peptide injection schedule was delayed until 65 min after anti-Fas antibody injection, i.e., at a time when apoptotic DNA fragmentation in the liver had already started (Fig. 2 *b*). Four out of the five Z-VAD–injected mice survived and one died after 10 h with a severely damaged liver.

Mice protected by this injection schedule started 5 min after antibody injection were then killed or bled at different times to follow the cysteine protease activity of their liver and their blood level of aminotransferases. Unexpectedly, the first wave of Z-YVAD-AFP cleavage activity of the hepatocyte cytosol was not decreased (Fig. 2 *c*, *left*); in contrast, the Z-DEVD-AFP cleavage activity was decreased and had returned to baseline levels after 90 min, at a time when unprotected mice showed a very high activity (Fig. 2 *c*, *right*). In agreement with this last observation, no cleavage of CPP32 was detected on Western blots (Fig. 3). Blood aminotransferase levels showed only a very small increase compared with that of unprotected mice and were at the baseline levels 4 d later (Fig. 2 *a*). Histologic examination of the liver obtained at various times of sacrifice 1 to several days later showed no signs of damage, indicating full protection. In contrast, one of the four mice surviving after the 65-min–delayed Z-VAD injection schedule mentioned above and sacrificed 1 d later showed histologically focal areas of liver necrosis; these lesions can certainly heal, because the three other surviving mice of this group were in apparent good health in the following weeks. The histologic appearance of various organs (heart, spleen, gut, lung, brain, intestine) was also normal, suggesting that the injected peptides, at the dosage used, had no toxic effect. It should be added that protection of the liver in all these experiments cannot be attributed to an unexpected effect of a Z-xmk compound on hepatocytes, since mice injected with the same amount of the Z-D.cmk compound died, as did control mice, with identical lesions.

A more specific inhibitor of cysteine proteases of the ICE subfamily is the tetrapeptide Ac-YVAD.cmk. In cell culture, it is, however, more difficult to inhibit Fas-mediated apoptosis with this peptide. Using mouse Fas–transfected W4 and P815 cell lines, we have observed that Fas-mediated apoptosis, which is otherwise almost complete within 4 h, is highly inhibited at concentration of 100 μM by Z-VAD.-fmk, but only partially inhibited in the presence of 300–600 μM Ac-YVAD.fmk, perhaps because of the lesser cell permeability of the latter compound. Two mice were injected with Ac-YVAD.cmk according to the schedule described above, but with a double amount (i.e., initial dose of 0.5 mg): one mouse died after 7.5 h and the other survived (but its liver was not examined). Because the intracellular availability of the peptide may be slower, the peptide administration schedule was applied to one mouse starting 15 min before anti-Fas injection; this mouse survived and had no gross or histologic liver lesions when sacrificed 1 d later. In spite of their small number, these last results conclusively show that administration of a peptide of more restricted anti-ICE specificity is capable of preventing Fas-mediated apoptosis in vivo.

Discussion

The cysteine protease activity triggered in hepatocytes in vivo by anti-Fas antibody injections showed a pattern of sequential activity comparable to that observed during Fas-induced apoptosis of mouse W4 cells (9), with a transient ICE-like activity observed first, followed by a gradually increasing CPP32-like activity. However, whereas on Western blots some degree of cleavage of proICE was observed after 45 min and of proCPP32 after 90 min, these cleavages were much weaker than those observed on Western blots of W4 and P815 cells undergoing in vitro Fas-induced apoptosis (Ody, C., K. Matsuura, and P. Vassalli, unpublished observation). This observation suggests that other members of the Ced-3 cysteine proteases family may be more preferentially involved in hepatocyte PCD; the liver appears to be especially rich in ICE_{rel} II and III proteases (belonging to the ICE subfamily) and in Mch2 (considered to belong to the CPP32 subfamily), the cleavage of which could not be explored because of the lack of relevant antibodies. Some of these last proteases appear to be more susceptible to some protease inhibitors than other members of their subfamily (17). This may explain why hepatocytes may be protected by the Z-VAD and YVAD inhibitory peptides more easily than W4 or P815 cells, because it is not likely that the concentration of these peptides required for in vitro prevention of Fas-mediated apoptosis of these cell lines, as described above, had been durably achieved in vivo. The observation that ICE-like protease activity was not inhibited in the liver of the Z-VAD protected mice, in contrast to CPP32-like activity, might suggest that Z-VAD.fmk is a better inhibitor of the latter enzymes. However, because it has been observed that Z-VAD.fmk inhibits poorly in vitro CPP32-like proteolysis once activated (18), it is more likely that the failure of Z-VAD injections, with the injection schedule used, to efficiently inhibit the early ICE-like protease activity, in contrast to the late CPP32-like activity, results from an insufficiently high intracellular concentration of the inhibitory peptides after the first injection. The observation that injections of the Ac-YVAD.cmk peptide also completely prevented liver damage when started before anti-Fas antibody injection strongly supports the notion of a PCD-inducing proteolytic cascade that can be interrupted

at its early stages. However, the first element of the cascade in Fas-induced PCD is the activation of the MACH-FLICE cysteine protease, bound to the MORT(FADD)-Fas protein complex (10, 11). This protease, which belongs to the CPP32 subfamily, cleaves after its activation the Z-DEVD- but not the Z-YVAD-AFC peptide (10). Thus, the early autoamplification steps of the cysteine protease cascade, rather than the probable initial proteolytic event, appear to be the targets of the inhibitory peptides used to prevent hepatocyte death in these experiments.

An intriguing question raised by the obvious instability and rapid decrease in vivo of the inhibitory peptides is how full and permanent protection against liver damage and death can be achieved by a few hours of protection after injection of an amount as high as 10 μg of agonist antibody, which is not expected to diffuse very rapidly out of the vascular compartment. At least three not mutually exclusive possibilities must be considered, which are presently under study: rapid removal of the antibody by Fas molecules makes it drop to a level insufficient to trigger significant hepatocyte damage; the level of Fas molecules on hepatocytes may also drop and lead to the same result; protected hepatocytes may undergo metabolic changes making them more resistant to Fas-mediated death, as occurs with TNF-sensitive cells first exposed to a sublethal concentration of TNF (19).

The possibility of preventing or attenuating in vivo fulminant liver damage resulting from liver apoptosis has important therapeutic implications in humans. It is generally assumed that fulminant hepatitis occurring during viral hepatitis is mediated by cytotoxic T lymphocytes. The cytotoxic activity of these cells is mediated through the perforin–granzyme and/or the Fas pathway (20); the mechanisms of cell death are probably common in these two pathways because granzyme B, a serine protease with Asp specificity of cleavage, is an efficient activator of CPP32 (21–23). The temporary perfusion of peptides with a high cell permeability designed to be potent irreversible inhibitors of the ICE-like proteases (24, 25) may thus be of great therapeutic benefit if these compounds show no general toxicity. In the very limited pattern of injection used in the present experiments, no signs of toxicity were observed; Z-VAD.fmk in vitro at a concentration of 100 μM has been found not to be toxic for at least 48 h (26). Our observation that Z-VAD.fmk injections protected mice from massive liver destruction and death even when delayed until a time when apoptotic DNA cleavage was detectable strengthens the potential therapeutic value of this approach. A number of other acute clinical situations are probably accompanied by cell death, especially harmful in tissues where, in contrast to hepatocytes, regeneration does not occur, such as the nervous tissue; in vitro, Z-VAD.fmk has been found to protect neurons from certain apoptotic-inducing conditions (27). Finally, perfusion of cysteine protease inhibitory peptides may be also of experimental use to detect in vivo the involvement of PCD in physiological or pathological conditions, as well as to explore the mechanism underlying various PCD, because in vitro not all PCD-inducing conditions display sensitivity to inhibitory peptides such as Z-VAD.fmk.

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