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# Multiple Pathways of TWEAK-Induced Cell Death<sup>1</sup>

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TWEAK, a recently identified member of the TNF family, is expressed on IFN- $\gamma$ -stimulated monocytes and induces cell death in certain tumor cell lines. In this study, we characterized the TWEAK-induced cell death in several tumor cell lines that exhibited distinct features. Although the TWEAK-induced cell death in Kym-1 cells was indirectly mediated by TNF- $\alpha$  and was inhibited by cycloheximide, the TWEAK-induced cell death in HSC3 cells or IFN- $\gamma$ -treated HT-29 cells was not inhibited by anti-TNF- $\alpha$  mAb or cycloheximide, suggesting a direct triggering of cell death via TWEAK receptor in the latter cell lines. The TWEAK-induced apoptosis in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells was associated with caspase-8 and caspase-3 activation. Although a pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, inhibited the TWEAK-induced cell death in HSC3 cells, it rather sensitized HT-29 cells to TWEAK-induced cell death by necrosis. This necrosis was abrogated by lysosomal proteinase inhibitors, particularly a cathepsin B inhibitor, [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester. During the process of TWEAK-induced necrosis, cathepsin B was released from lysosome to cytosol. Although DR3 has been reported to be a receptor for TWEAK, all TWEAK-sensitive tumor cell lines used in this study did not express DR3 at either protein or mRNA level, but did bind CD8-TWEAK specifically. These results indicated that TWEAK could induce multiple pathways of cell death, including both caspase-dependent apoptosis and cathepsin B-dependent necrosis, in a cell type-specific manner via TWEAK receptor(s) distinct from DR3. *The Journal of Immunology*, 2002, 168: 734–743.

Several members of the TNF family, such as TNF- $\alpha$ , Fas ligand (FasL)<sup>3</sup>/CD95 ligand/APO-1 ligand, and TNF-related apoptosis-inducing ligand (TRAIL)/APO-2 ligand, induce cell death in a variety of tumor cells and nontransformed cells and are critically involved in tumor suppression, homeostasis of immune system, and pathogenesis of various diseases (1–3). TWEAK has recently been identified as a new death-inducing ligand belonging to the TNF family (4). It has also been reported that TWEAK induces proliferation of endothelial cells and angiogenesis (5). Although TWEAK mRNA has been found in various tissues and cells (4, 6), its expression at the protein level and its

physiological role remain largely unknown. We have recently demonstrated that TWEAK is expressed on human peripheral blood (PB) monocytes upon IFN- $\gamma$  stimulation and is involved in IFN- $\gamma$ -stimulated monocyte cytotoxicity against TWEAK-sensitive tumor cells (7). However, the TWEAK-induced death signaling pathway has not been well characterized.

So far, the mechanisms of TNF- $\alpha$ , FasL-, or TRAIL-induced cell death have been well characterized. These death-inducing ligands exert their cytotoxic effects through TNF-R1, Fas, TRAIL-R1/death receptor (DR)4, and TRAIL-R2/DR5, respectively. Trimeric ligand-induced oligomerization of the cytoplasmic death domain (DD) of these receptors leads to recruitment of caspase-8 via Fas-associated DD or a Fas-associated DD-like adaptor molecule, which activates the caspase cascade, resulting in apoptosis (8–10). In some cell types, TNF- $\alpha$  and FasL could induce apoptosis not only via the activation of caspases, but also via the activation of noncaspase proteinases such as cathepsins and calpains (11–14). Furthermore, TNF- $\alpha$  and FasL could also induce necrosis, which appears to be mediated by production of reactive oxygen intermediates and/or loss of mitochondrial transmembrane potential ( $\Delta\psi_m$ ; Refs. 15–18).

It has been reported that TWEAK induced cell death through DR3/TRAMP/LARD/APO-3/WSL1 (6). DR3 is a type I membrane protein belonging to the TNF-R family, and it contains the DD homologous to TNF-R1, Fas, TRAIL-R1, and TRAIL-R2 (19–21). However, there are conflicting reports as to the TWEAK-DR3 interaction. For example, Schneider et al. (22) have reported that TWEAK could induce cell death in Kym-1 cells lacking DR3 mRNA expression. In addition, Kaptein et al. (23) have reported that TWEAK could not bind to DR3 in an in vitro binding assay. Thus, it remains unclear whether DR3 is really involved in TWEAK-induced cell death. Furthermore, Schneider et al. (22) have demonstrated that the TWEAK-induced cell death in Kym-1 cells was indirectly mediated by the interaction of endogenous TNF- $\alpha$  and TNF-R1. Thus, it also remains unclear whether

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<sup>3</sup> Abbreviations used in this paper: FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; h, human; TWEAK-R, TWEAK receptor; PB, peripheral blood; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-4-methyl-coumaryl-7-amide; Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-4-methyl-coumaryl-7-amide; Ac-YVAD-MCA, acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide; CHX, cycloheximide; BHA, butylated hydroxyanisole; CA074 Me, [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester; L-NMMA, L-monomethyl-L-arginine monoacetate; L-NIO, N-(1-iminoethyl)-L-ornithine; WST, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl) [2-<sup>3</sup>H]tetrazolium monosodium salt; sFasL, soluble FasL; RIP, receptor-interacting protein; DD, death domain.

TWEAK receptor (TWEAK-R) could directly induce cell death, like TNF-R1 and Fas.

In the present study, we characterized the mechanisms of TWEAK-induced cell death in several tumor cell lines. We found that TWEAK could directly induce apoptosis via caspase activation in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells, and that TWEAK could also induce necrosis via a lysosomal cathepsin B pathway when caspases were blocked by benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) in IFN- $\gamma$ -treated HT-29 cells. In addition, DR3 expression was not detectable in all TWEAK-sensitive tumor cell lines examined in this study. These results indicated that TWEAK could directly induce both apoptosis and necrosis via death-inducing receptor(s) distinct from DR3.

## Materials and Methods

### Cells

Human colon adenocarcinoma HT-29 cells and human T cell lymphoma Jurkat cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 containing 10% FCS, 100  $\mu$ g/ml streptomycin and penicillin, and 2 mM glutamine (culture medium). Human oral squamous cell carcinoma HSC3 cells and human gastric adenocarcinoma KATO-III cells were obtained from Japan Cancer Research Bank (Osaka, Japan) and were maintained in the culture medium. Human rhabdomyosarcoma Kym-1 cells were kindly provided by Dr. H. Endo (Ichi Medical School, Tochigi, Japan) and were cultured in DMEM containing 10% FCS, 100  $\mu$ g/ml streptomycin and penicillin, and 2 mM glutamine.

### Reagents

Human IFN- $\gamma$  and anti-human TNF- $\alpha$  mAb (mAb1) were purchased from BD Pharmingen (San Diego, CA). z-VAD-fmk and [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA074 Me) were purchased from Peptide Institute (Osaka, Japan). Boc-Asp-fluoromethylketone was purchased from Calbiochem (San Diego, CA). Anti-human Fas mAb (CH-11) and recombinant soluble human FasL were purchased from MBL (Nagoya, Japan) and Alexis (San Diego, CA), respectively. Butylated hydroxyanisole (BHA), L-monomethyl-L-arginine monoacetate (L-NMMA), *N*-(1-iminoethyl)-L-omithine (L-NIO), and Bafilomycin A1 were purchased from Wako Pure Chemicals (Osaka, Japan). D609 was purchased from Calbiochem. Desipramine was purchased from Sigma-Aldrich (St. Louis, MO). CD8-human TWEAK fusion protein was prepared as described previously (7). An anti-human DR3 mAb (JD3, mouse IgG1/ $\kappa$ ) was generated by immunizing a BALB/c mouse with human (h)DR3-Ig fusion protein and screening for binding to hDR3-transfected BHK (hDR3/BHK) and L5178Y (hDR3/L5178Y) cells (described elsewhere).

### Cell viability assay

Cells ( $5 \times 10^3$  or  $1 \times 10^4$  per well) were cultured with or without the indicated dose of CD8-TWEAK, anti-Fas mAb, or TNF- $\alpha$  in the presence or absence of IFN- $\gamma$  (20 ng/ml) for the indicated period in a flat-bottom 96-well microtiter plate. In some experiments, cells were pretreated with IFN- $\gamma$  (20 ng/ml) for 12 h and/or z-VAD-fmk (50  $\mu$ M) for 1 h before the CD8-TWEAK treatment. In some experiments, to inhibit new protein synthesis, cells were pretreated with cycloheximide (CHX; 2  $\mu$ g/ml) for 1 h before the CD8-TWEAK treatment. This dose of CHX was not toxic for the cells, but it could inhibit new protein synthesis by at least 86% in the cells used in this study, as estimated by the uptake of  $^35$ S-methionine into proteins. Cell viability was then determined by measuring the metabolic activity using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl) [2H]tetrazolium monosodium salt (WST; Wako Pure Chemicals) or the standard crystal violet assay as described previously (7).

### Fluorogenic substrate assay for caspase activity

The activity of caspases was measured as described by Vercammen et al. (16) with minor modifications. After various treatments, cells ( $1 \times 10^6$ ) were resuspended in the lysis buffer (0.5% Nonidet P-40, 250 mM NaCl, 50 mM Tris-HCl, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 100  $\mu$ M PMSF). The lysates were centrifuged at  $15,000 \times g$  for 15 min, and the supernatants were collected. The extracts (40  $\mu$ g of total protein) were incubated in 100  $\mu$ l of the cell-free system buffer (10 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM EGTA, 2 mM  $\text{MgCl}_2$ , 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT)

with 100  $\mu$ M of the fluorogenic peptide substrates acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), acetyl-Ile-Glu-Thr-Asp-4-methyl-coumaryl-7-amide (Ac-IETD-MCA), acetyl-Leu-Glu-His-Asp-4-methyl-coumaryl-7-amide (Ac-LEHD-MCA), or acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide (Ac-YVAD-MCA; Peptide Institute) to measure caspase-3-, -8-, -9-, or -1-like activity, respectively. The release of fluorescent aminomethylcoumarin was measured for 1 h at 5-min intervals on a Fluoroskan Ascent (Labsystems, Helsinki, Finland). Data are expressed as the increase in fluorescence as a function of time.

### Western blot analysis for caspase activation

HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells ( $1 \times 10^6$ ) were incubated with or without CD8-TWEAK (100 ng/ml) for 6 or 24 h, respectively. Then the cells were lysed in 200  $\mu$ l of the lysis buffer. The extracts (30  $\mu$ g of total protein) were subjected to 15% SDS-PAGE for caspase-3 and p18 processed form of caspase-8, or 12% SDS-PAGE for pro-form and p41, p43 processed forms of caspase-8. After blotting onto polyvinylidene difluoride membranes, the caspase fragments were detected with rabbit anti-human caspase-3 Ab (kindly provided by Drs. R. Takahashi and J. C. Reed, Burnham Institute, La Jolla, CA), mouse anti-human caspase-8 mAb (5F7, MBL), or mouse anti- $\alpha$ -tubulin mAb (Oncogene Science, Cambridge, MA) and then developed by ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ).

### Measurement of DNA hypoploidy by flow cytometry

DNA hypoploidy was measured as described by Nicoletti et al. (24) with minor modifications. In brief, cells ( $1 \times 10^6$ ) were stained in 900  $\mu$ l of 50  $\mu$ g/ml propidium iodide in 0.1% Triton X-100, 4 mM sodium citrate (pH 7.2), and 450  $\mu$ g/ml RNase for 10 min at 4°C, and then 100  $\mu$ l of 1.5 M NaCl was added. Samples were incubated in the dark at 4°C for 30 min and then analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). Data were processed by using the CellQuest program (BD Biosciences).

### Electron microscopy

After various treatments, cells ( $3 \times 10^6$ ) were fixed with 2% glutaraldehyde in PBS for 2 h and then with 2%  $\text{OsO}_4$  for 2 h before embedding in Epon 812. Thin sections were prepared using a MT-5000 ultramicrotome (DuPont Pharmaceuticals, Wilmington, DE), stained with uranyl acetate followed by lead citrate, and then observed on a JEM1230 electron transmission microscope (JEOL, Tokyo, Japan).

### Subcellular fractionation and Western blot analysis for cathepsin B

HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells ( $2 \times 10^7$ ) were incubated with z-VAD-fmk (50  $\mu$ M) and CD8-TWEAK (100 ng/ml) for the indicated periods. Cytosolic fractions (S-100) were prepared as described by Yang et al. (25) with minor modifications. Cell pellets were resuspended in 400  $\mu$ l of 0.25 M sucrose containing 5 mM sodium phosphate buffer (pH 7.2). The cells were homogenized by passing them through a 23-gauge needle 60 times, followed by two rounds of centrifugation at  $750 \times g$  for 10 min at 4°C to remove the nuclei and unbroken cells. The supernatants (enucleated whole-cell lysates) were then centrifuged at  $10,000 \times g$  for 30 min at 4°C to remove lysosomes and mitochondria. The resulting supernatants were further centrifuged at  $100,000 \times g$  for 1 h at 4°C. The final supernatants (cytosolic fractions) were collected and then concentrated by 5% TCA, including 0.02% deoxycholic acid precipitation. The cytosolic fractions (30  $\mu$ g) and the whole-cell lysates (30  $\mu$ g) were subjected to 12% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and probed with rabbit anti-cathepsin B Ab (26) or mouse anti- $\alpha$ -tubulin mAb (Oncogene Science), followed by detection with ECL Plus (Amersham Pharmacia Biotech).

### Flow cytometric analysis for DR3 and TWEAK-R expression

Cells ( $1 \times 10^6$ ) were incubated with 0.5  $\mu$ g of CD8-TWEAK or biotinylated anti-human DR3 mAb for 1 h at 4°C, followed by PE-labeled anti-human CD8 mAb (BD Pharmingen) or PE-labeled avidin (BD Pharmingen), respectively. After washing with PBS, the cells were analyzed on a FACSCalibur, and data were analyzed by using the CellQuest program.

### Northern blot analysis for DR3 mRNA

Northern blot analysis was performed as described previously (27). In brief, tumor cells were cultured in the presence or absence of IFN- $\gamma$  (20  $\mu$ g/ml) for 12 h. Total RNA was extracted from the cells by using RBA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instruction. Ten micrograms each of denatured RNA was electrophoresed

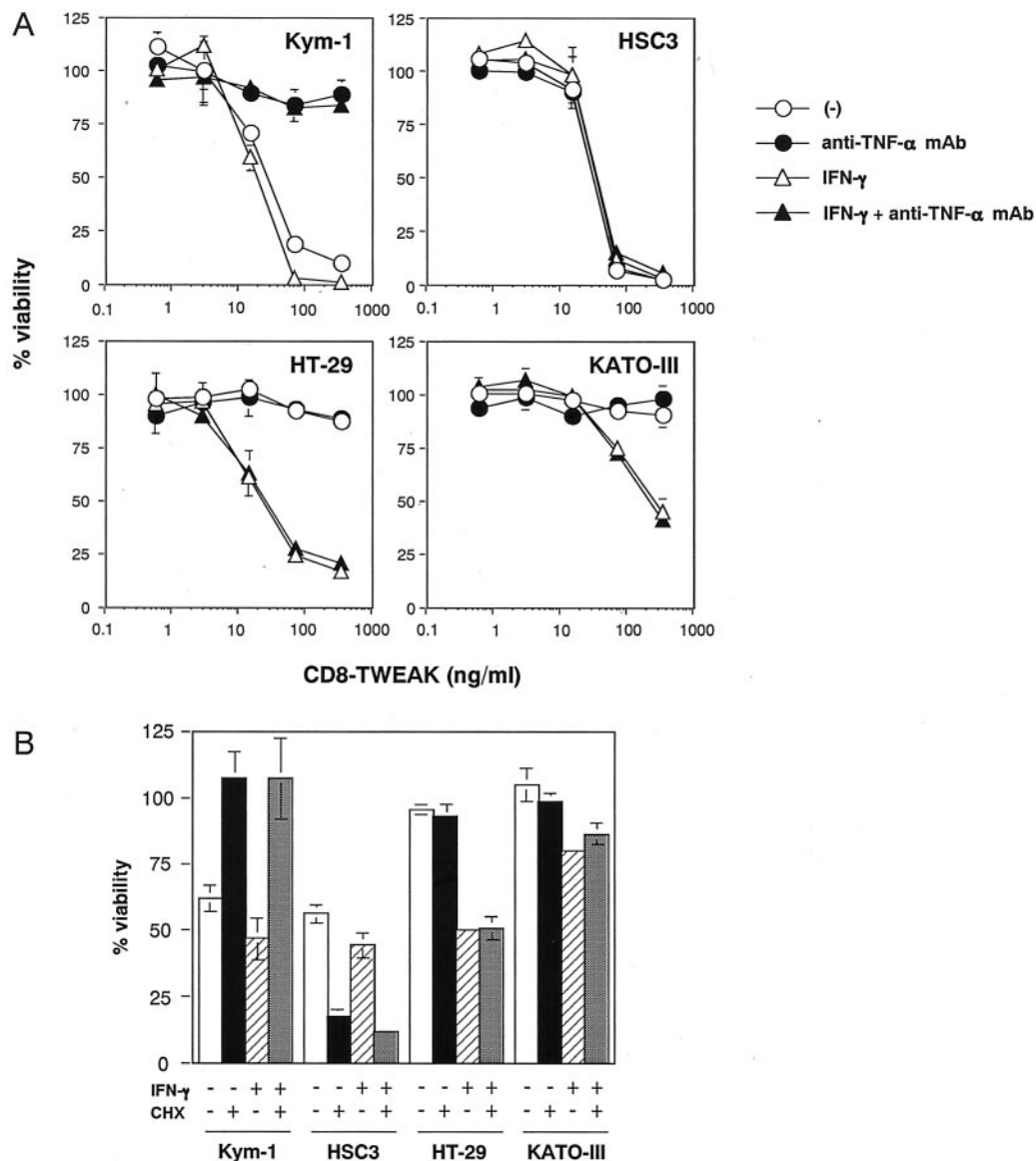
in a 1.5% agarose gel containing 6.6% formaldehyde and then blotted onto a nylon membrane. The membrane was hybridized with an [ $\alpha$ - $^{32}$ P]dCTP-labeled 1100-bp *Bam*HI/*Xba*I fragment containing human TRAMP/DR3 cDNA (kindly provided by Dr. J. Tschopp, Lausanne University, Epalinges, Switzerland) at 65°C in ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA), and then washed twice in 2× SSC/0.1% SDS at 65°C for 15 min. The autoradiogram was analyzed on a BAS2500 (Fuji Film, Tokyo, Japan).

## Results

### Distinct features of TWEAK-induced cell death among tumor cell lines

We and others have shown that TWEAK induced cell death in certain tumor cell lines in the presence or absence of IFN- $\gamma$  (4, 7). Recently, Schneider et al. (22) have reported that the TWEAK-induced cell death in Kym-1 cells was indirectly mediated by the interaction of endogenous TNF- $\alpha$  and TNF-R1. Thus, we first

tested whether TWEAK-induced cell death was generally mediated by endogenous TNF- $\alpha$  in several TWEAK-sensitive tumor cell lines. As previously reported (4, 7, 22), Kym-1 and HSC3 cells were sensitive to TWEAK in the absence of IFN- $\gamma$ , and HT-29 and KATO-III cells were sensitive to TWEAK in the presence of IFN- $\gamma$  (Fig. 1A). The TWEAK-induced cell death in all these cell lines was specifically abrogated by a neutralizing anti-human TWEAK mAb CARL-1 (Ref. 7 and data not shown). Consistent with the observation by Schneider et al. (22), the CD8-TWEAK-induced cell death in Kym-1 cells was completely inhibited by neutralizing anti-TNF- $\alpha$  mAb. In contrast, anti-TNF- $\alpha$  mAb did not inhibit the TWEAK-induced cell death in HSC3, HT-29, or KATO-III cells (Fig. 1A). To further examine whether endogenous FasL or TRAIL might be involved in the TWEAK-induced cell death in HSC3, HT-29, and KATO-III cells, we tested the effect of neutralizing anti-FasL (NOK-1; Ref. 28) and anti-TRAIL (RIK-2;



**FIGURE 1.** Characterization of TWEAK-induced cell death in tumor cell lines. **A**, Effect of anti-TNF- $\alpha$  mAb on TWEAK-induced cell death. The indicated tumor cells ( $5 \times 10^3$ ) were cultured with the indicated concentrations of CD8-TWEAK in the presence or absence of IFN- $\gamma$  (20 ng/ml) and/or anti-TNF- $\alpha$  mAb (10  $\mu$ g/ml). After a 36-h culture, viability was estimated by the WST assay. Data are represented as the mean  $\pm$  SD of triplicate samples. Similar results were obtained in three independent experiments. **B**, Effect of CHX on TWEAK-induced cell death. The indicated tumor cells ( $1 \times 10^4$ ) were cultured with CD8-TWEAK (100 ng/ml) in the presence or absence of IFN- $\gamma$  (20 ng/ml) and/or CHX (2  $\mu$ g/ml). After a 24-h culture, viability was estimated by the WST assay. Data are represented as the mean  $\pm$  SD of triplicate samples. Similar results were obtained in three independent experiments.

Ref. 29) mAbs on the TWEAK-induced cell death in these cells. However, the mixture of anti-TNF- $\alpha$ , anti-FasL, and anti-TRAIL mAbs did not significantly inhibit the TWEAK-induced cell death in HSC3, HT-29, and KATO-III cells (data not shown). These results indicated that the TWEAK-induced cell death was not generally mediated indirectly by endogenous TNF- $\alpha$ , FasL, or TRAIL.

Because we could not rule out a possible contribution of endogenously induced death factor(s) other than TNF- $\alpha$ , FasL, and TRAIL, we next examined the effect of a protein synthesis inhibitor CHX on TWEAK-induced cell death. As shown in Fig. 1B, the CHX treatment abrogated the TWEAK-induced cell death in Kym-1 cells, but not in HSC3, HT-29, or KATO-III cells. The TWEAK-induced cell death in HSC3 cells was rather enhanced by the CHX treatment. These results suggested that TWEAK could induce cell death directly via its receptor on HSC3, HT-29, and KATO-III cells independently of de novo synthesis of an endogenous death factor.

#### Activation of caspases by TWEAK

The death-inducing ligands such as TNF- $\alpha$ , FasL, and TRAIL can induce apoptosis via activation of caspases (1, 2, 8–10). However, it remains unknown whether TWEAK could activate caspases. Thus, we next investigated whether caspases were activated in the course of TWEAK-induced cell death in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells. We also examined anti-Fas- or TNF- $\alpha$ -induced cell death in these cells for comparison. HSC3 cells were sensitive to TWEAK and anti-Fas mAb, but not TNF- $\alpha$  (Fig. 2A). HT-29 cells were sensitive to TWEAK, anti-Fas mAb, and TNF- $\alpha$  only when pretreated with IFN- $\gamma$  (Fig. 2A). We then examined caspase activities in the cell lysates by using fluorogenic peptide substrates, Ac-DEVD-MCA, Ac-IETD-MCA, Ac-LEHD-MCA, and Ac-YVAD-MCA for caspase-3, -8, -9, and -1, respectively. In HSC3 cells, anti-Fas mAb rapidly activated caspase-3, -8, and -9, which peaked at 3 h (Fig. 2B). TWEAK also activated these caspases with somewhat delayed kinetics, which peaked at 6 h. In HT-29 cells, anti-Fas mAb rapidly activated caspase-3, -8, and -9, which peaked at 3 h (Fig. 2B). In contrast, TWEAK and TNF- $\alpha$  activated caspase-3 and -8 with greatly delayed and sustained kinetics, which gradually increased from 6 to 36 h. TWEAK, but not TNF- $\alpha$ , activated caspase-9 significantly. These differences in kinetics of caspase activation were mostly correlated with rapid kinetics of TWEAK- or anti-Fas-induced cell death in HSC3 cells, which reached a plateau within 12 h, and slower kinetics of TWEAK- and TNF- $\alpha$ -induced cell death in HT-29 cells, which gradually increased over 36 h (Fig. 2A).

To further identify the caspases activated by TWEAK in HSC3 and HT-29 cells, we subjected the cell lysates of HSC3 and HT-29 cells after the treatment with CD8-TWEAK for 6 and 24 h, respectively, to Western blot analysis with anti-caspase-3 and -8 Abs. It has been shown that 36-kDa human procaspase-3 is cleaved at Asp<sup>175</sup>, resulting in a 24-kDa large subunit, which is further cleaved at either Asp<sup>9</sup> or Asp<sup>28</sup>, resulting in p20 or p17 large subunit, respectively (30). We used an anti-human caspase-3 Ab (31), which recognizes 36-kDa pro-form, and p24, p20, and p17 processed forms to assess caspase-3 activation by cleavage. Human procaspase-8 has been shown to be 54–55 kDa and cleaved into p18 protein through intermediate p41 and p43 (32). We used an anti-human caspase-8 mAb, which recognize the pro-form and p43, p41, and p18 processed forms to assess caspase-8 activation by cleavage. As shown in Fig. 2C, TWEAK stimulation could induce processing of 36-kDa procaspase-3 into p20 and p17 in HSC3 cells, and into p24 and p20 in HT-29 cells. A substantial part of procaspase-8 was processed into p18 in TWEAK-stimulated HSC3 cells, and into p43, p41, and p18 in TWEAK-stimu-

lated HT-29 cells (Fig. 2C). Taken together, these results indicated that TWEAK could activate caspase-8 and caspase-3 in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells.

#### Effect of z-VAD-fmk on TWEAK-induced cell death and apoptosis

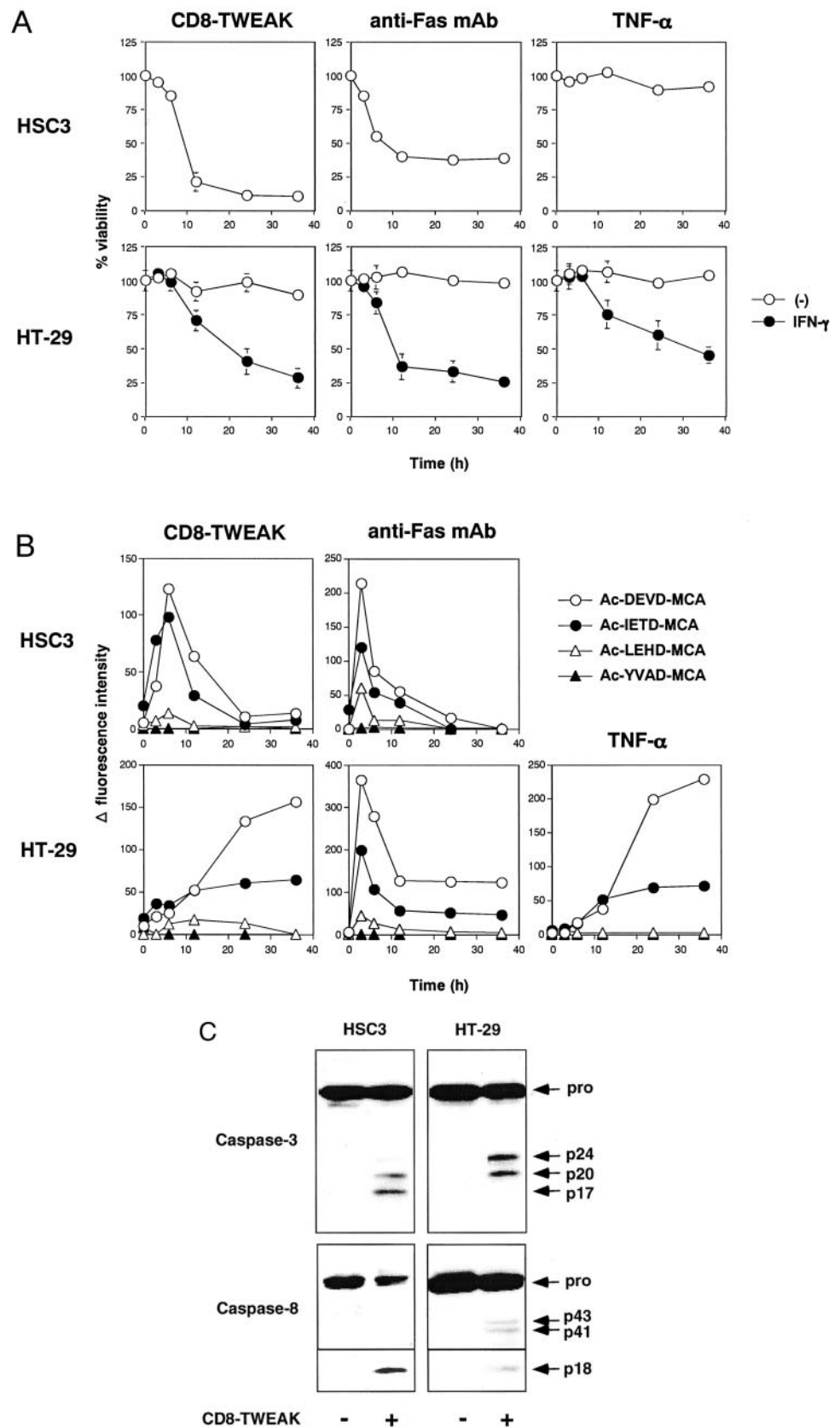
To assess whether the TWEAK-induced cell death was dependent on the caspase activation, we next examined the effect of a pan-caspase inhibitor, z-VAD-fmk, on the TWEAK-, anti-Fas-, or TNF- $\alpha$ -induced cell death in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells. In addition, we also examined whether TWEAK could induce DNA hypoploidy as an indication of apoptosis in a caspase-dependent manner. As shown in Fig. 3, A and B, both cell death and DNA hypoploidy were blocked by z-VAD-fmk in TWEAK- or anti-Fas-stimulated HSC3 cells, suggesting that the TWEAK-induced HSC3 cell death was primarily due to caspase-dependent apoptosis with nuclear disintegration like that caused by Fas-mediated signaling. In HT-29 cells, z-VAD-fmk also blocked both cell death and DNA hypoploidy when stimulated by anti-Fas mAb or TNF- $\alpha$  (Fig. 3, A and B). Recently, it has been reported that z-VAD-fmk abrogated apoptosis, but not cell death, in T cell lines induced by a high dose of cross-linked recombinant soluble FasL (sFasL) or TNF- $\alpha$  (18, 33). However, in HT-29 cells, z-VAD-fmk abrogated both cell death and apoptosis even when stimulated with a high dose of sFasL (1000 ng/ml), anti-Fas mAb (5000 ng/ml), or TNF- $\alpha$  (1000 ng/ml; data not shown). In contrast, z-VAD-fmk rather markedly enhanced TWEAK-induced cell death (Fig. 3A), despite efficiently blocking DNA hypoploidy (Fig. 3B). Similar results were obtained with another pan-caspase inhibitor, Boc-Asp-fluoromethylketone (data not shown). These results indicated that caspase inhibition could abrogate sFasL-, anti-Fas mAb-, or TNF- $\alpha$ -induced cell death, but rather enhanced TWEAK-induced cell death in HT-29 cells.

To further characterize the TWEAK-induced HSC3 and HT-29 cell death, we next examined the morphology of dying cells by transmission electron microscopy. As shown in Fig. 4, the TWEAK-induced HSC3 cell death was associated with typical apoptotic morphological changes such as chromatin condensation, cell shrinking, and apoptotic bodies, like anti-Fas mAb-induced apoptosis. These changes were completely abrogated by z-VAD-fmk. In HT-29 cells, TWEAK, anti-Fas mAb, and TNF- $\alpha$  also induced apoptotic morphological changes. These changes induced by anti-Fas mAb and TNF- $\alpha$  were completely abrogated by z-VAD-fmk. Notably, the TWEAK-stimulated HT-29 cells in the presence of z-VAD-fmk exhibited a necrotic morphology, which was characterized by loss of plasma membrane integrity without apparent damage to nuclei. These results indicated that TWEAK could primarily induce apoptosis in HSC3 and HT-29 cells, but it could also induce necrosis in HT-29 cells when caspases were inactivated.

#### Involvement of cathepsin B in TWEAK-induced necrosis

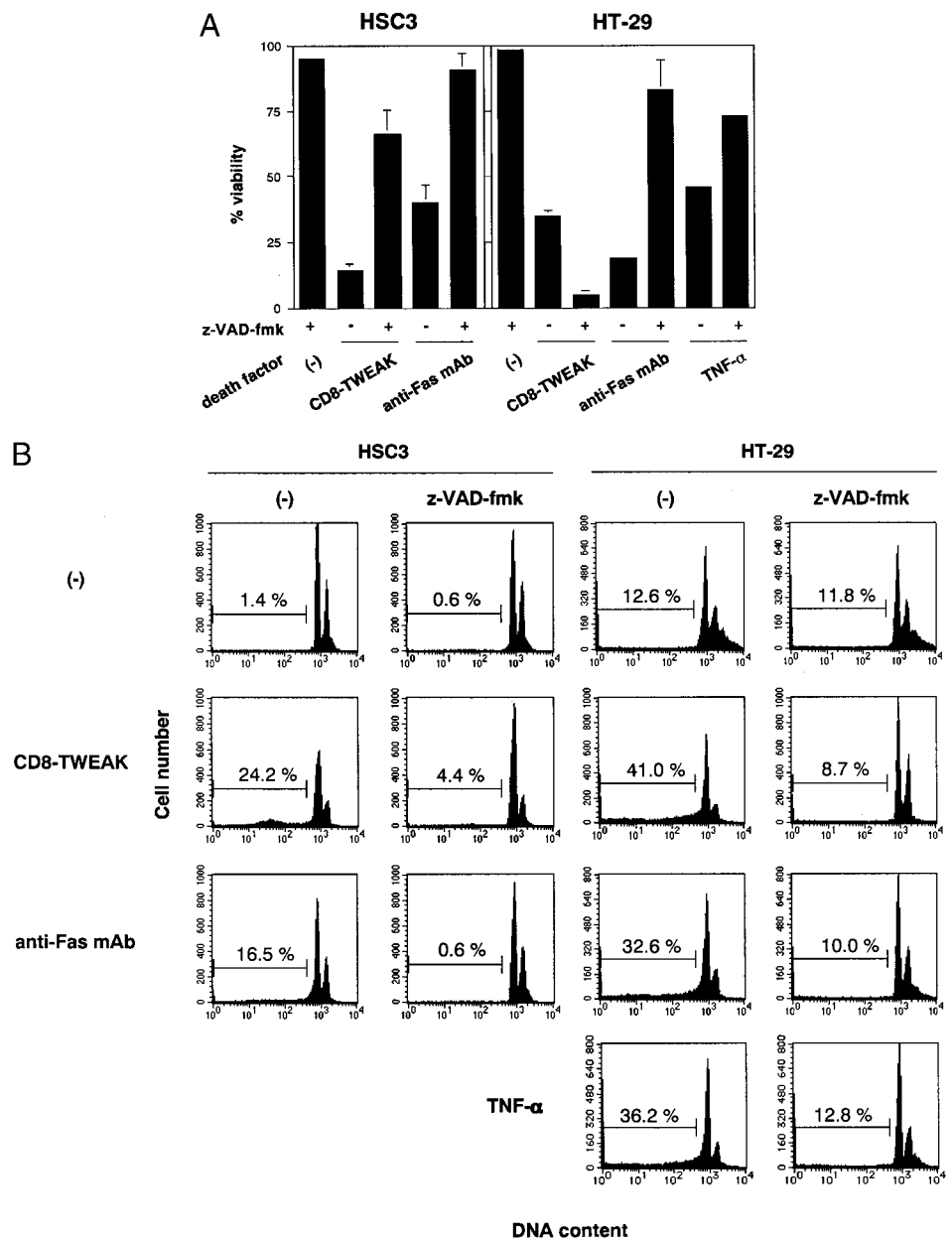
In contrast to apoptosis, signaling pathways leading to necrosis have not been well characterized. Recently, it has been reported that mitochondrial stress-induced reactive oxygen intermediates were responsible for TNF- $\alpha$ - or anti-Fas mAb-induced necrosis, which was abrogated by antioxidants such as BHA (15–17). Thus, we tested whether BHA could inhibit the TWEAK-induced necrosis in z-VAD-fmk-treated HT-29 cells. Although BHA completely inhibited the TNF- $\alpha$ -induced necrosis in mouse fibrosarcoma L929 cells (data not shown) as previously reported (16), it did not inhibit the TWEAK-induced necrosis in HT-29 cells, which was characterized by cell death without DNA hypoploidy (Fig. 5A). Moreover, we could not observe apparent morphological changes of

**FIGURE 2.** Activation of caspases by TWEAK, anti-Fas mAb, or TNF- $\alpha$  stimulation. *A*, Kinetics of TWEAK-, anti-Fas-, or TNF- $\alpha$ -induced cell death in HSC3 cells and IFN- $\gamma$ -pretreated HT-29 cells. HSC3 cells and untreated or IFN- $\gamma$  (20 ng/ml, 12 h)-pretreated HT-29 cells ( $5 \times 10^3$ ) were cultured with CD8-TWEAK (100 ng/ml), anti-Fas mAb (CH-11, 250 ng/ml), or TNF- $\alpha$  (50 ng/ml) for the indicated periods and viability was estimated by the crystal violet assay. Data are represented as the mean  $\pm$  SD of triplicate samples. Similar results were obtained in three independent experiments. *B*, Caspase activity in the cell lysate. HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were cultured with CD8-TWEAK (100 ng/ml), anti-Fas mAb (CH-11, 250 ng/ml), or TNF- $\alpha$  (50 ng/ml) for the indicated periods. Caspase-3-, -8-, -9-, and -1-like activities in the cell lysate (40  $\mu$ g) were measured by using Ac-DEVD-MCA, Ac-IETD-MCA, Ac-LEHD-MCA, and Ac-YVAD-MCA, respectively, as the substrates. Data are represented as the mean of triplicate samples (SD were  $< 10\%$  of the means; not shown). Similar results were obtained in three independent experiments. *C*, Western blot analysis of caspases. HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were cultured with CD8-TWEAK (100 ng/ml) for 6 and 24 h, respectively. The total cell lysate (30  $\mu$ g) was subjected to SDS-PAGE and immunoblotting with Abs specific for caspase-3 and -8 and  $\alpha$ -tubulin. The positions of pro-enzyme (pro) and processed fragments are indicated by arrows at the right. Similar results were obtained in three independent experiments.



mitochondria by electron microscopy or release of cytochrome *c* from mitochondria to cytosol by Western blot analysis in the necrotic HT-29 cells (data not shown). These results suggested that mitochondria stress might not be critically responsible for the

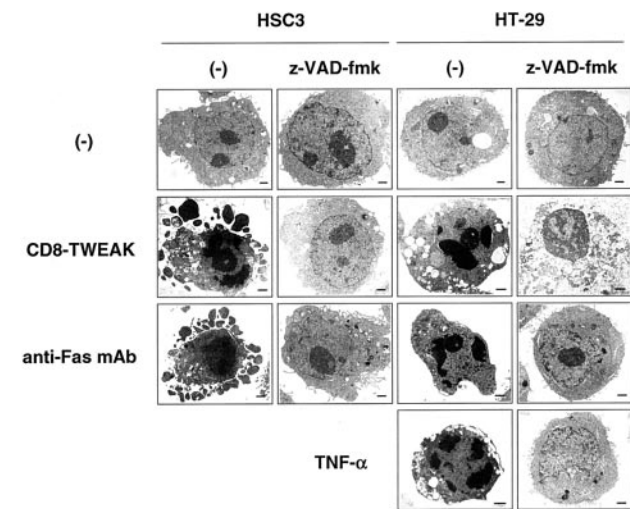
TWEAK-induced necrosis. It has also been reported that oxidative stress, such as NO, and sphingolipids, such as ceramide and sphingosine, could provoke necrosis (34, 35). Thus, we next examined the effect of various compounds, including inducible NO synthase



**FIGURE 3.** Effect of z-VAD-fmk on TWEAK-, anti-Fas mAb-, or TNF- $\alpha$ -induced cell death and DNA hypoploidy. HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were cultured with CD8-TWEAK (100 ng/ml), anti-Fas mAb (CH-11, 250 ng/ml), or TNF- $\alpha$  (50 ng/ml) in the presence or absence of z-VAD-fmk (50  $\mu$ M). After a 36-h culture, viability was estimated by the WST assay (A), and DNA hypoploidy was estimated by flow cytometry (B). Data are represented as the mean  $\pm$  SD of triplicate samples (A). Similar results were obtained in three independent experiments.

inhibitors, L-NMMA and L-NIO, and acidic sphingomyelinase inhibitors, desipramine and D609, on the TWEAK-induced necrosis. As shown in Fig. 5A, the TWEAK-induced necrosis was not significantly blocked by these inhibitors. It has also been reported that lysosomal stresses, such as autophagy or release of lysosomal enzymes, were involved in necrosis (36, 37). Furthermore, it has recently been reported that a lysosomal cysteine proteinase, cathepsin B, was involved in TNF- $\alpha$ -induced cell death (11, 12). Thus, we next examined the effect of a specific inhibitor of cathepsin B, CA074 Me (38), on the TWEAK-induced necrosis. As shown in Fig. 5A, CA074 Me almost completely abrogated the TWEAK-induced necrosis in HT-29 cells. It has been reported that pretreatment with a vacuolar H<sup>+</sup>-ATPase inhibitor, Bafilomycin A1, raised lysosomal pH, resulting in degradation of lysosomal proteinases, including cathepsin B (39). We observed that the 12-h pretreatment with Bafilomycin A1 also completely abrogated the TWEAK-induced necrosis in HT-29 cells (Fig. 5A). These results suggested that cathepsin B played a critical role in the TWEAK-induced necrosis in HT-29 cells.

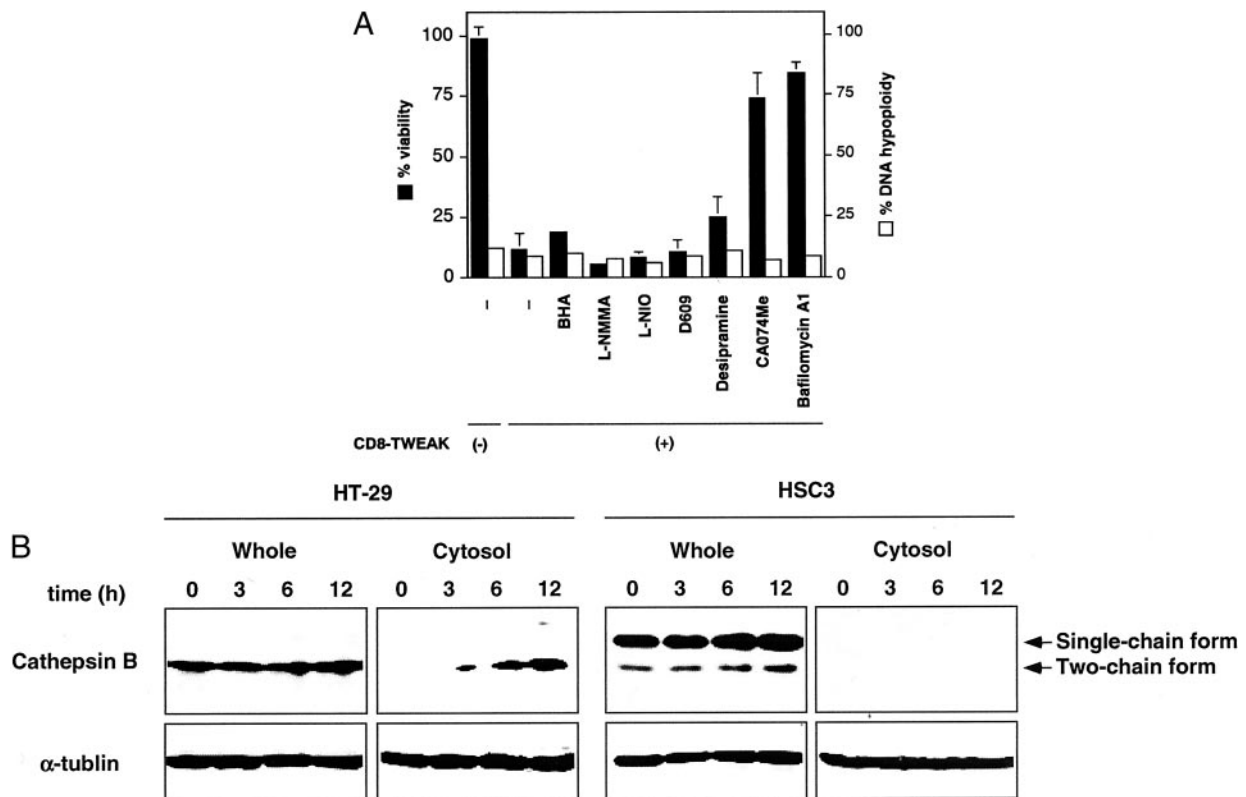
We further examined whether TWEAK could increase the amount of cathepsin B or induce the release of cathepsin B from lysosome to cytosol in HT-29 cells. It is known that cathepsin B is synthesized as a proenzyme and is transported into lysosomes where it is processed into active forms, a two-chain form (25–27 kDa) and single-chain form (29–31 kDa), by lysosomal cysteine proteinases (39, 40). As shown in Fig. 5B, we observed the only two-chain form of the mature cathepsin B in the whole cell lysate of HT-29 cells by Western blot analysis, and the TWEAK stimulation did not increase the amount of cathepsin B. Subcellular fractionation showed that a substantial amount of the two-chain form of the mature cathepsin B was released to cytosol in the TWEAK-stimulated HT-29 cells (Fig. 5B). In HSC3 cells that did not undergo TWEAK-induced necrosis, we observed both single-chain form and two-chain form of cathepsin B in the whole-cell lysate. However, these active forms of cathepsin B could not be released to cytosol by TWEAK stimulation (Fig. 5B). These results suggested that the TWEAK-induced necrosis was mediated by cathepsin B released from lysosomes.



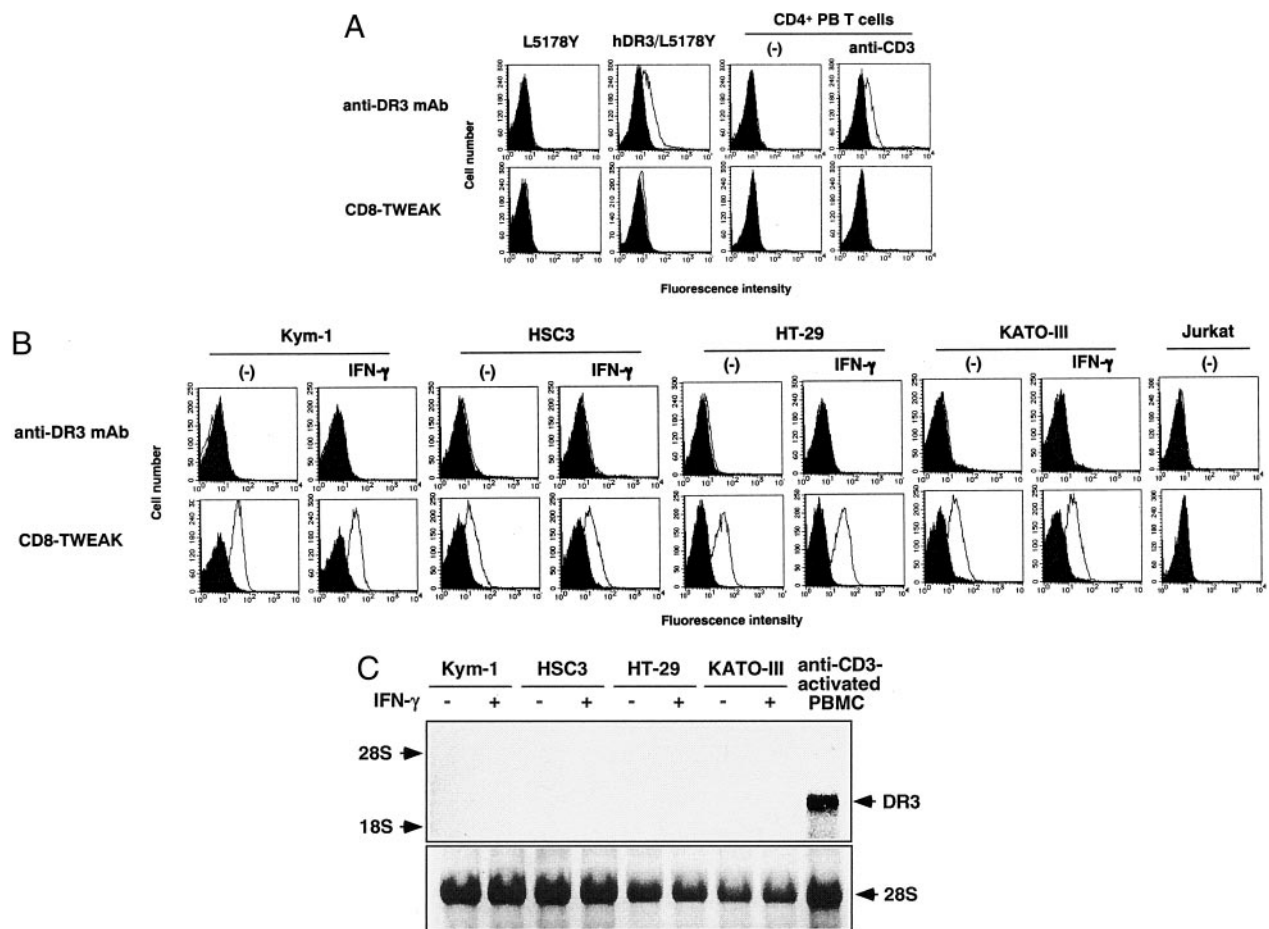
**FIGURE 4.** Morphology of TWEAK-, anti-Fas mAb-, or TNF- $\alpha$ -induced cell death and effect of z-VAD-fmk. HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were cultured with CD8-TWEAK (100 ng/ml), anti-Fas mAb (CH-11, 250 ng/ml), or TNF- $\alpha$  (50 ng/ml) in the presence or absence of z-VAD-fmk (50  $\mu$ M). After a 6-h (HSC3) or 12-h (HT-29) culture, the cells were observed under electron transmission microscope. The scale bars represent 1  $\mu$ m. Similar results were obtained in three independent experiments.

#### Expression of TWEAK-R and DR3 in TWEAK-sensitive tumor cell lines

It has been reported that TWEAK bound to and induced cell death via DR3 (6). However, there are conflicting results as to the TWEAK-DR3 interaction (23). To determine the involvement of DR3 in the TWEAK-induced cell death observed in the present study, we finally estimated the expression of DR3 on Kym-1, HSC3, HT-29, and KATO-III cells by cell surface staining with an anti-human DR3 mAb (JD3) that we recently established. This mAb was generated against hDR3-Ig fusion protein and specifically reacted with hDR3-transfected L5178Y (hDR3/L5178Y) cells (Fig. 6A) and hDR3/BHK cells (not shown). It also reacted with anti-CD3-stimulated PB T cells, but not with unstimulated PB T cells (Fig. 6A). As shown in Fig. 6B, this anti-DR3 mAb did not react with Kym-1, HSC3, HT-29, or KATO-III cells even after IFN- $\gamma$  pretreatment. It has been reported that DR3 has various splicing variant forms (21). Because we could not rule out the possibility that the anti-DR3 mAb we used could not recognize a certain splicing variant form of DR3, we further examined DR3 mRNA expression in these cell lines by Northern blot analysis. As shown in Fig. 6C, the expression of DR3 mRNA was not detectable in these tumor cell lines. We next examined the expression of TWEAK-R on these cell lines by binding of CD8-TWEAK detected by PE-labeled anti-human CD8 mAb. As shown in Fig. 6B, all the TWEAK-sensitive tumor cell lines, including Kym-1, HSC3, HT-29, and KATO-III, but not TWEAK-resistant Jurkat cells, bound CD8-TWEAK irrespective of IFN- $\gamma$  pretreatment.



**FIGURE 5.** Involvement of cathepsin B in TWEAK-induced necrosis. **A**, Effect of various inhibitors on TWEAK-induced necrosis. IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were precultured with BHA (100  $\mu$ M), L-NMMA (100  $\mu$ M), L-NIO (100  $\mu$ M), D609 (50  $\mu$ g/ml), Desipramine (50  $\mu$ g/ml), or CA074 Me (50  $\mu$ M) for 1 h, or Bafilomycin A1 (10 nM) for 12 h, and they were then cultured with z-VAD-fmk (50  $\mu$ M) and CD8-TWEAK (100 ng/ml). After a 12-h culture, viability (filled bars) and DNA hypoploidy (open bars) were estimated by the crystal violet assay and flow cytometry, respectively. Data are represented as the mean  $\pm$  SD of triplicate samples. Similar results were obtained in three independent experiments. **B**, Release of cathepsin B to cytosol. HSC3 cells and IFN- $\gamma$  (20 ng/ml)-treated HT-29 cells were cultured with z-VAD-fmk (50  $\mu$ M) and CD8-TWEAK (100 ng/ml) for the indicated periods. Whole-cell lysate (30  $\mu$ g) and cytosolic fraction (30  $\mu$ g) were subjected to SDS-PAGE and immunoblotting with Abs specific for cathepsin B and  $\alpha$ -tubulin. Similar results were obtained in three independent experiments.



**FIGURE 6.** Expression of DR3 and TWEAK-R on tumor cell lines. *A*, Cell surface staining of DR3 transfectants and human PB T cells with anti-DR3 mAb and CD8-TWEAK. L5178Y, hDR3/L5178Y, and human PBMCs cultured for 12 h with or without anti-CD3 mAb were stained with biotinylated anti-hDR3 mAb or CD8-TWEAK, followed by PE-labeled avidin plus FITC-labeled anti-CD4 mAb or PE-labeled anti-CD8 mAb, respectively (open histograms). Filled histograms indicate background staining with biotinylated control IgG plus PE-labeled avidin or PE-labeled anti-CD8 mAb alone. Similar results were obtained in three independent experiments. *B*, Cell surface staining of tumor cell lines with anti-DR3 mAb and CD8-TWEAK. The indicated TWEAK-sensitive tumor cell lines before and after treatment with IFN- $\gamma$  (20 ng/ml) and a TWEAK-resistant Jurkat cell line were stained as described in *A*. Similar results were obtained in three independent experiments. *C*, Northern blot analysis of DR3 mRNA expression in the tumor cell lines. Total RNAs were extracted from the indicated tumor cells and anti-CD3-activated PBMC, and 10  $\mu$ g of each was subjected to Northern blot with hDR3 cDNA probe. Similar results were obtained in two independent experiments.

The binding of CD8-TWEAK to these cell lines was specific because it was abrogated by preincubation with a neutralizing anti-TWEAK mAb, CARL-1 (data not shown). These results suggested that the TWEAK-induced cell death in these cell lines was mediated by TWEAK-R other than DR3. It should be also noted that CD8-TWEAK did not bind to hDR3/L5178Y cells or anti-CD3-stimulated PB T cells expressing DR3 on cell surface, suggesting that DR3 might not be a receptor for TWEAK.

## Discussion

In this study, we investigated the mechanisms of TWEAK-induced cell death in several tumor cell lines that exhibited distinct features. Although the TWEAK-induced cell death in Kym-1 cells was indirectly mediated by endogenous TNF- $\alpha$ , TWEAK could directly induce apoptosis in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells by activating caspase-8 and caspase-3, like anti-Fas mAb and TNF- $\alpha$ . The pan-caspase inhibitor z-VAD-fmk abrogated both the TWEAK-induced apoptosis and cell death in HSC3 cells. In HT-29 cells, z-VAD-fmk abrogated the TWEAK-induced apoptosis characterized by DNA hypoploidy and chromatin condensation, but was sensitized to death by necrosis via a cathepsin B pathway. In addition, no detectable levels of DR3, which has been reported

to be a receptor for TWEAK, were observed in all the TWEAK-sensitive tumor cell lines tested in this study. These results are the first indication that TWEAK could directly induce not only the caspase-dependent apoptosis but also the cathepsin B-dependent necrosis in a cell type-specific manner via an as-yet-undefined receptor(s) distinct from DR3.

A recent study by others (22) has demonstrated that the TWEAK-induced cell death was indirectly mediated by the interaction of endogenous TNF- $\alpha$  and TNF-R1 in Kym-1 cells lacking DR3. It has also been reported that TNF-R2-, CD30-, or CD40-mediated cell death was indirect and dependent on de novo synthesis of endogenous death-inducing ligands such as TNF- $\alpha$ , FasL, and TRAIL in some tumor cell lines (41, 42). These results suggested that the putative TWEAK-R other than DR3 might be a non-DD-containing receptor like CD30 and CD40. However, in this study, we observed that the TWEAK-induced cell death in HSC3 cells and IFN- $\gamma$ -treated HT-29 cells could not be inhibited by neutralizing anti-TNF- $\alpha$  mAb, anti-FasL mAb, anti-TRAIL mAb, or CHX. Moreover, TWEAK induced caspase-8 and caspase-3 activation with similar kinetics to anti-Fas mAb or TNF- $\alpha$  in these cells. These results strongly suggested that

TWEAK could directly induce cell death via a DD-containing receptor other than DR3.

Recently, we have reported that TWEAK was expressed on IFN- $\gamma$ -stimulated monocytes and that it contributed to their cytotoxicity against TWEAK-sensitive tumor cells (7). As shown in Fig. 1A, TWEAK exerted cytotoxic activity against some tumor cell lines only in the presence of IFN- $\gamma$ . A previous study in the murine system indicated a critical role for monocytes in tumor rejection *in vivo* that was evoked by endogenous IFN- $\gamma$  (43). In this respect, endogenous IFN- $\gamma$  might exert a synergistic antitumor effect by up-regulating the expression of TWEAK on tumor-infiltrating monocytes and by sensitizing tumor cells to TWEAK-induced cell death *in vivo*. However, the mechanism by which IFN- $\gamma$  sensitizes the tumor cells to TWEAK is still unclear. IFN- $\gamma$  has been reported to modulate cell death by inducing several apoptosis-related genes, including TNF-R1, Fas, and caspase-1 (44, 45). However, we could not observe either the up-regulation of TWEAK-R (Fig. 6B) or the TWEAK-induced caspase-1 activation (Fig. 2B) in IFN- $\gamma$ -treated HT-29 cells. It has been reported that inhibition of NF- $\kappa$ B activation sensitized some tumor cells to TNF- $\alpha$ -, TRAIL-, or FasL-induced cell death (46). Thus, it is possible that IFN- $\gamma$  sensitized HT-29 cells to TWEAK-induced cell death by inhibiting TWEAK-induced NF- $\kappa$ B activation. We observed that TWEAK could activate NF- $\kappa$ B in HT-29 cells, but IFN- $\gamma$  did not affect the NF- $\kappa$ B activity (data not shown). In HT-29 cells, IFN- $\gamma$  was essential not only for TWEAK-induced apoptosis, but also for necrosis (data not shown), suggesting that IFN- $\gamma$  modulates a common signaling component in apoptosis and necrosis. Further studies are now under way to determine the molecular mechanism for the IFN- $\gamma$  action in TWEAK-induced apoptosis and necrosis.

In this study, we found that the inhibition of caspases by z-VAD-fmk sensitized IFN- $\gamma$ -treated HT-29 cells to TWEAK-induced necrosis. A similar sensitization to the TWEAK-induced necrosis by z-VAD-fmk was also observed in IFN- $\gamma$ -treated colon cancer cell lines, SW620 and Colo205 (our unpublished data). The signaling pathways leading to necrosis are not well understood. We revealed that cathepsin B was responsible for the TWEAK-induced necrosis in HT-29 cells. It is well known that cathepsin B is a lysosomal cysteine proteinase that contributes to proteolysis of proteins taken up by phagocytosis. Recently, some papers have shown that cathepsin B is involved in TNF- $\alpha$ -induced cell death (11, 12). For example, Guicciardi et al. (11) have reported that cathepsin B contributed to TNF- $\alpha$ -induced hepatocyte apoptosis and that cathepsin B-deficient mice were resistant to TNF- $\alpha$ -mediated hepatitis. More recently, Foghsgaard et al. (12) have reported that cathepsin B acted as an essential downstream mediator of TNF-induced and caspases-mediated apoptosis in several tumor cell lines. In this study, we demonstrated that cathepsin B was responsible for TWEAK-induced necrosis independently of caspase activation. Moreover, we demonstrated that cathepsin B was released to cytosol by TWEAK stimulation. However, target molecules for the released cathepsin B leading to necrosis remains unknown. Guicciardi et al. (11) have reported that cathepsin B could increase the release of cytochrome *c* from mitochondria via unknown cytosolic factor in a cell-free system. However, we could not observe the cytochrome *c* release from mitochondria to cytosol during the TWEAK-induced necrosis in HT-29 cells (data not shown). Further biochemical analysis will be needed to identify the target molecule(s) cleaved by cathepsin B and leading to necrosis. Recently, Holler et al. (33) have reported that FasL-, TRAIL-, or TNF- $\alpha$ -induced necrosis was mediated by receptor-interacting protein (RIP). We also observed that the TWEAK-induced necrosis could be abrogated by 1  $\mu$ g/ml geldanamycin (our unpublished

data), which has been reported to degrade RIP by disruption of heat-shock protein 90 interacting with RIP (33, 47). However, further studies are needed to determine the involvement of RIP in TWEAK-induced necrosis in HT-29 cells.

A previous study has shown that TWEAK could induce cell death via DR3 (6). It has been reported that DR3 expression was observed preferentially in lymphocytes (19, 20) and is rapidly up-regulated in human PB lymphocytes upon stimulation with PMA plus IL-2 (20). In this study, we demonstrated that cell surface expression of DR3 was induced on anti-CD3 mAb-stimulated PB T cells (Fig. 6A). These results suggested that DR3 might regulate activated T cells. Consistent with this notion, a recent study using DR3-deficient mice has revealed that DR3 was involved in anti-CD3-induced thymocyte apoptosis and negative selection during thymocyte development (48). Thus, TWEAK might regulate activated T cells via DR3. However, we could not observe CD8-TWEAK binding to anti-CD3-stimulated PB T cells (Fig. 6A). In addition, CD8-TWEAK did not induce either cell death or proliferation in anti-CD3 mAb-stimulated PB T cells (our unpublished data). Moreover, we could not observe CD8-TWEAK binding to DR3 transfectants (Fig. 6A). Similar results have been obtained by Kaptein et al. (23). These results suggest that TWEAK is not a physiological ligand for DR3. Conversely, we could not detect the expression of DR3 in all the TWEAK-sensitive tumor cell lines used in this study at the protein or mRNA levels, while they did bind CD8-TWEAK (Fig. 6). This suggests that TWEAK could induce cell death via a TWEAK-R distinct from DR3.

The putative TWEAK-R remains to be identified. Our present results indicate that the TWEAK-R expressed on HSC3 cells acts like Fas to rapidly activate caspase-8 and caspase-3 (Fig. 2B), leading to cell death by apoptosis (Figs. 3 and 4). In contrast, the TWEAK-R expressed on HT-29 cells acts like TNF-R1 to slowly activate caspase-8 and caspase-3 (Fig. 2B), leading to cell death primarily by apoptosis (Figs. 3 and 4). However, the z-VAD-fmk treatment did not induce necrosis in TNF- $\alpha$ -stimulated HT-29 cells, indicating a unique feature of TWEAK-R, leading to the cathepsin B-mediated necrosis. It is also worth noting that the TWEAK-R expressed on Kym-1 cells did not induce cell death directly because the TWEAK-induced cell death in Kym-1 cells was completely abrogated by anti-TNF- $\alpha$  mAb (Fig. 1A). It remains to be determined whether these distinct features of TWEAK-induced cell death are mediated by distinct TWEAK-Rs or whether a single TWEAK-R transmits differential signals in particular cellular contexts. Further studies are now under way to address these possibilities.

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