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Fibroblast Growth Factor-Inducible 14 Mediates Multiple Pathways of TWEAK-Induced Cell Death

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TWEAK, a TNF family member, is produced by IFN-γ-stimulated monocytes and induces multiple pathways of cell death, including caspase-dependent apoptosis, cathepsin B-dependent necrosis, and endogenous TNF-α-mediated cell death, in a cell type-specific manner. However, the TWEAK receptor(s) that mediate these multiple death pathways remains to be identified. Recently, fibroblast growth factor-inducible 14 (Fn14) has been identified to be a TWEAK receptor, which was responsible for TWEAK-induced proliferation of endothelial cells and angiogenesis. Because Fn14 lacks the cytoplasmic death domain, it remains unclear whether Fn14 can also mediate the TWEAK-induced cell death. In this study, we demonstrated that TWEAK could induce apoptotic cell death in Fn14 transfectants. A pan-caspase inhibitor, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone, rather sensitized the Fn14 transfectants to TWEAK-Induced cell death by necrosis via reactive oxygen intermediates and cathepsin B-dependent pathway. By using newly generated agonistic anti-Fn14 mAbs, we also observed that Fn14 is constitutively expressed on the cell surface of all TWEAK-sensitive tumor cell lines, and can transmit the multiple death signals. Moreover, an anti-Fn14 mAb that blocks TWEAK-Fn14 interaction could totally abrogate TWEAK binding and TWEAK-induced cell death in all TWEAK-sensitive tumor cell lines. These results revealed that the multiple pathways of TWEAK-induced cell death are solely mediated by Fn14. The Journal of Immunology, 2003, 170: 341–348.

Members of the TNF family play important roles in regulating various cellular responses, including proliferation, differentiation, and death (1, 2). TWEAK, a member of the TNF family, induces cell death in some tumor cell lines (3, 4), but also induces proliferation of human endothelial cells in vitro and angiogenesis in vivo (5). We have recently reported that TWEAK is expressed on IFN-γ-stimulated human monocytes and is involved in their cytotoxicity (6). We and others have also demonstrated that TWEAK could induce multiple pathways of cell death in different cellular contexts (4, 7). In Kym-1 cells, TWEAK-induced cell death was indirectly mediated by endogenously produced TNF-α, like that mediated by other TNFR family members lacking the death domain (DD)† such as TNFR2, CD30, and CD40 (8, 9). In HSC3 cells and IFN-γ-treated HT-29 cells, TWEAK could directly induce apoptosis via caspase activation (7), like that mediated by DD-containing TNFR family members such as TNFR1, Fas, TNF-related apoptosis-inducing ligand (TRAIL)-R1/DR4, and TRAIL-R2/DR5 (1, 2). In HSC3 cells, a pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) abrogated both the TWEAK-induced apoptosis and cell death (7). In HT-29 cells, z-VAD-fmk abrogated the TWEAK-induced apoptosis, but rather sensitized to death by necrosis via a lysosomal cathepsin B pathway (7). These TWEAK-sensitive tumor cell lines did not express DR3/TRAMP/LARD/APO-3/WSL1 (4, 7), which is a DD-containing TNFR family member (10, 11) previously reported to be a receptor for TWEAK (12). These results suggested the existence of death-inducing TWEAK receptor(s) distinct from DR3, which remains to be identified. It also remains to be determined whether these distinct modes of TWEAK-induced cell death are mediated by distinct TWEAK receptors or whether a single TWEAK receptor transmits differential signals in particular cellular contexts.

Recently, Wiley et al. (13) have identified a TWEAK receptor from HUVEC cDNA library by expression cloning using soluble rTWEAK. The TWEAK receptor turned out to be a fibroblast growth factor-inducible 14-kDa protein, Fn14, which was originally identified by a differential display approach to search for growth factor-inducible molecules in murine NIH3T3 fibroblasts (14, 15). Fn14 is a distantly related TNFR family member, which contains only one cysteine-rich domain in the extracellular region and a TNFR-associated factor (TRAF) binding domain, but not the DD, in the cytoplasmic region (13). Wiley et al. (13) have reported that cross-linking of Fn14 could induce proliferation of HUVEC, and that soluble Fn14 inhibited epidermal growth factor-induced endothelial cell migration in vitro and fibroblast growth factor-2-induced angiogenesis in vivo. These results suggested that Fn14 is responsible for the TWEAK-induced endothelial cell migration, proliferation, and angiogenesis. However, it remains unclear whether Fn14 is also responsible for the TWEAK-induced cell
death in tumor cell lines we observed. In this study, we demonstrated that TWEAK could induce cell death in Fn14 transfectants and that the multiple pathways of TWEAK-induced cell death in several tumor cell lines were solely mediated by Fn14.

**Materials and Methods**

**Cells**

Human colon adenocarcinoma HT-29, human T lymphoma Jurkat, human hepatoma SK-Hep-1, and mouse T lymphoma L5178Y were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine (culture medium). Human oral squamous cell carcinoma HSC3 and mouse mastocytoma P815 were obtained from Japan Cancer Research Bank (Osaka, Japan) and maintained in the culture medium. Human rhabdomyosarcoma Kym-1 was kindly provided by H. Endo (Ichi Medical School, Tochigi, Japan) and cultured in DMEM containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine. HUVEC was obtained from Clonetics (San Diego, CA) and cultured in endothelial cell growth medium-2.

**Reagents**

Human IFN-γ and anti-human TNF-α mAb (mAb1) were purchased from BD PharMingen (San Diego, CA). z-VAD-Fmk and a cathepsin B-specific inhibitor, Ac-Leu-Glu-His-Asp-4-methyl-coumaryl-7-amide (Ac-LEHD-MCA), or acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide (Ac-TVAD-MCA; Peptide Institute) to measure caspase-3-, -8,-9, and -1-like activity, respectively. The release of fluorescent aminomethylcoumarin was measured for 1 h at 5-min intervals on a Fluoroscan Ascent (Labsystems, Helsinki, Finland). Data are expressed as the increase in fluorescence as a function of time.

**Cell viability assay**

Cells (5 × 10^5/well) were cultured with or without the indicated doses of CD8-TWEAK or anti-Fn14 mAbs for the indicated period in a flat-bottom 96-well microtiter plate. For HT-29, IFN-γ (20 ng/ml) was included. In some experiments, cells were pretreated with z-VAD-fmk (10 or 50 μM), BHA (100 μM), and/or CA074 Me (10 μM) for 1 h before the CD8-TWEAK treatment. The cell viability was then determined by measuring the metabolic activity using 2-(4-iophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-3H-tetrazolium salt (WST; Wako Pure Chemicals), as described previously (6). The viability was calculated as follows: (A_{untreated} - A_{treated}) / A_{untreated} × 100.

**Fluorogenic substrate assay for caspase activity**

Activity of caspases was measured, as described previously (7). After CD8-TWEAK treatment, cells (1 × 10^6) were resuspended in the lysis buffer (0.5% Nonidet P-40, 250 mM NaCl, 50 mM Tris-HCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM PMSF). The lysates were centrifuged at 15,000 × g for 15 min, and the supernatants were collected. The extracts (40 μg total protein) were incubated in 100 μl of the cell-free system buffer (10 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM DTT) with 100 μM of the fluorogenic peptide substrates acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVAD-MCA), acetyl-Leu-Glu-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), acetyl-Leu-Glu-Asp-4-methyl-coumaryl-7-amide (Ac-LEHD-MCA), or acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide (Ac-TVAD-MCA; Peptide Institute) to measure caspase-3,-8,-9, and -1-like activity, respectively. The release of fluorescent aminomethylcoumarin was measured for 1 h at 5-min intervals on a Fluoroscan Ascent (Labsystems, Helsinki, Finland). Data are expressed as the increase in fluorescence as a function of time.
by a neutralizing anti-human TWEAK mAb CARL-1 (6) (data not shown). These results confirmed that Fn14 acts as a TWEAK receptor.

Recently, Wiley et al. (13) have reported that TWEAK could induce proliferation of endothelial cells via Fn14. We also observed that CD8-TWEAK we used could bind to HUVEC (Fig. 1A) and induce proliferation of HUVEC in a low-serum condition (Fig. 1B). Thus, we first examined whether CD8-TWEAK could induce the proliferation of hFn14/L5178Y and hFn14/P815 cells in a low (1%) or high (10%) FCS condition. As shown in Fig. 1B, both hFn14/L5178Y and hFn14/P815 cells did not proliferate, but rather progressively lost viability in response to CD8-TWEAK in a dose-dependent manner in the 1% FCS condition, which was completely reversed by the neutralizing anti-TWEAK mAb. This suggested that TWEAK could induce cell death in these hFn14 transfectants. Because hFn14/L5178Y cells exhibited a higher sensitivity to CD8-TWEAK than hFn14/P815 cells (Fig. 1B), we used hFn14/L5178Y cells to further characterize the TWEAK-induced cell death in Fn14 transfectants. As represented in Figs. 1C and 2A, hFn14/L5178Y cells, but not parental L5178Y cells, started to die at 24 h in the 10% FCS condition. Neither a transcription inhibitor actinomycin D nor a translation inhibitor cycloheximide affected the level or kinetics of cell death (data not shown), suggesting that TWEAK could induce cell death in hFn14/L5178Y cells independently of de novo protein synthesis.

To explore whether Fn14-mediated cell death is dependent on caspase activation, we next examined the effect of a pan-caspase inhibitor z-VAD-fmk on TWEAK-induced cell death in hFn14/L5178Y cells. As shown in Fig. 1C, z-VAD-fmk, which alone was not toxic for the cells, markedly accelerated and enhanced the TWEAK-induced cell death, which was completely inhibited by anti-TWEAK mAb CARL-1. Similar results were obtained with another pan-caspase inhibitor, Boc-D-fmk (data not shown), but not vehicle (DMSO) alone. To date, we and others have observed a similar enhancing effect of z-VAD-fmk on TWEAK-, TNF-α-, or anti-Fas mAb-induced cell death in certain tumor cell lines, which was mediated by ROI or lysosomal cathepsin B, and thus abrogated by antioxidants such as BHA or a cathepsin B inhibitor CA074 Me (7, 20–22). As shown in Fig. 2A, the TWEAK-induced cell death in hFn14/L5178Y cells either in the presence or absence of z-VAD-fmk was also significantly inhibited by both BHA and CA074 Me. These results suggested the involvement of ROI and cathepsin B in the Fn14-mediated cell death, which was negatively regulated by z-VAD-fmk-sensitive caspase(s). To investigate whether caspses were activated in hFn14/L5178Y cells by stimulation with CD8-TWEAK, we measured caspase activities in the cell lysate 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-like activities, respectively. As a control, we also measured caspase activities in IFN-γ-treated

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**FIGURE 1.** Characterization of hFn14 transfectants. **A,** Cell surface staining with CD8-TWEAK. L5178Y, hFn14/L5178Y, P815, hFn14/P815 cells, and HUVEC were stained with CD8-TWEAK, followed by PE-labeled anti-human CD8 mAb (open histograms) or PE-labeled anti-human CD8 mAb alone (filled histograms). **B,** Effect of CD8-TWEAK on the growth of hFn14 transfectants and HUVEC. Cells were cultured with the indicated doses of CD8-TWEAK in the presence or absence of CARL-1 (5 μg/ml) in a low-serum condition for 5 days. Relative cell number was determined by the WST assay. Data represent mean ± SD of triplicate samples. Similar results were obtained in two independent experiments. **C,** Kinetics of TWEAK-induced cell death in hFn14/L5178Y cells. hFn14/L5178Y cells were cultured with CD8-TWEAK (500 ng/ml) in the absence or presence of CARL-1 (5 μg/ml) and/or z-VAD-fmk (10 μM) for the indicated periods. Percentage of dead cell was determined by propidium iodide staining and flow cytometry. Data represent mean ± SD of triplicate samples. Similar results were obtained in two independent experiments.
HT-29 cells after TWEAK stimulation. As we previously reported (7), substantial levels of caspase-3- and -8-like activities were induced in HT-29 cells upon CD8-TWEAK stimulation (Fig. 2B). In hFn14/L5178Y cells, only a marginal level of caspase-3-like activity was detected (Fig. 2B). The levels of caspase activation in these cells were correlated with their sensitivity to TWEAK-induced cell death (Fig. 2B).

We previously observed that the TWEAK-induced cell death in HT-29 cells in the absence or presence of z-VAD-fmk showed apoptotic or necrotic morphology, respectively (7). Likewise, the TWEAK-treated hFn14/L5178Y cells in the absence or presence of z-VAD-fmk also exhibited apoptotic morphology characterized by condensed and fragmented nucleus or necrotic morphology characterized by cell swelling, respectively. The necrotic morphology was completely abrogated by BHA or CA074 Me (Fig. 2C). These results indicated that TWEAK could primarily induce apoptotic death in hFn14/L5178Y cells, and that z-VAD-fmk could sensitize hFn14/L5178Y cells to TWEAK-induced cell death by necrosis via ROI- and cathepsin B-dependent pathway.

We next measured the intracellular ROI level in TWEAK-stimulated hFn14/L5178Y cells by flow cytometry using a fluorogenic substrate DHR123. As shown in Fig. 2D, the ROI level was markedly increased by CD8-TWEAK in the presence of z-VAD-fmk, with a peak at 24 h. The ROI increase was blocked by the pretreatment with BHA or CA074 Me, suggesting that cathepsin B activity was required for the maximal increase of intracellular ROI level in response to TWEAK.

We next examined whether TWEAK could induce the release of cathepsin B from lysosome to cytosol in the Fn14 transfectants. We previously demonstrated the cathepsin B release from lysosome in TWEAK-stimulated HT-29 cells undergoing necrosis (7). As shown in Fig. 2E, hFn14/L5178Y cells contained two active forms of cathepsin B, single-chain form and two-chain form, in the whole cell lysate, which were not increased by CD8-TWEAK stimulation. Subcellular fractionation showed that a substantial amount of the two-chain form of cathepsin B was present in the cytosol of TWEAK-stimulated hFn14/L5178Y cells. Pretreatment with CA074 Me, which specifically inhibits cathepsin B activity by masking active site cysteine residue (23), resulted in accumulation of the single-chain form in the cytosol (Fig. 2F), possibly due to blocking of autocatalytic processing to the two-chain form. This indicated that the cathepsin B activity per se was not required for the cathepsin B release. Notably, the TWEAK-induced cathepsin B release was totally abrogated by BHA (Fig. 2F). This suggested that ROI played a crucial role to induce the cathepsin B release from lysosome.

Recently, both lysosomal damage and oxidative stress have been implicated in some models of cell death (24–28). For example, cathepsin B was responsible for oxidative stress-induced cell death in neuronal cells (27). In contrast, p53-induced cell death involved early lysosomal damage, followed by mitochondrial damage, which was blocked by BHA (25). Cathepsin D release preceded ROI production in the course of retinoid-induced HL60 cell death (28). In TWEAK-stimulated hFn14/L5178Y cells, cathepsin B release was abrogated by antioxidant BHA (Fig. 2F), and ROI production was inhibited by cathepsin B inhibitor CA074 Me (Fig. 2D), while both BHA and CA074 Me effectively inhibited the TWEAK-induced cell death (Fig. 2, A and C). These results suggested that ROI and cathepsin B constituted a positive feedback loop in mediating the TWEAK-induced cell death in hFn14/L5178Y cells.

Because ROI have been linked with NF-κB activation (29), we also investigated whether TWEAK could activate NF-κB and whether z-VAD-fmk might affect the NF-κB activation in hFn14/L5178Y cells by Western blotting analysis for IκBα degradation, which reflects NF-κB activation (17). As shown in Fig. 2G, IκBα was degraded at 10 min, and then gradually reactivated from 30 to 60 min after CD8-TWEAK stimulation. However, z-VAD-fmk did not affect the level or kinetics of the IκBα degradation. These results suggested that TWEAK-induced cell death in hFn14/L5178Y cells was not critically regulated by NF-κB activity.

**Characterization of anti-hFn14 mAbs**

To further characterize the expression and function of Fn14, we generated four mAbs that specifically bind to hFn14 and exhibit cytotoxic activity against hFn14/L5178Y cells. Hybridomas were prepared from splenocytes from mice immunized with the hFn14/Ph15 cells. Four hybridomas producing ITEM-1 (IgG1/κ), ITEM-2 (IgA/κ), ITEM-3 (IgG2b/κ), and ITEM-4 (IgG2b/κ) mAbs were selected by their cytotoxicity against hFn14/L5178Y cells in the presence of z-VAD-fmk. As represented in Fig. 3A, all

![Figure 2](http://www.jimmunol.org/)
mAbs reacted with hFn14/L5178Y cells, but not with parental L5178Y cells, as estimated by cell surface staining. These mAbs exhibited cytotoxic activity against hFn14/L5178Y, but not L5178Y, cells in a dose-dependent manner in the presence of z-VAD-fmk (Fig. 3B). ITEM-1 exhibited the strongest cytotoxic effect even in the absence of z-VAD-fmk. The ITEM-1-induced cell death in hFn14/L5178Y cells showed apoptotic or necrotic morphology in the absence or presence of z-VAD-fmk, respectively, as estimated by electron microscopy (data not shown), as was the TWEAK-induced cell death (Fig. 2C). These results further substantiated that hFn14 expressed on L5178Y cells could induce both apoptotic and necrotic cell death.

Expression and function of Fn14 on human tumor cell lines

We next examined the expression and function of Fn14 on human tumor cell lines that undergo different modes of TWEAK-induced cell death. We previously demonstrated that TWEAK induced caspase-dependent apoptosis and cathepsin B-dependent necrosis in HT-29 cells, only caspase-dependent apoptosis in HSC3 cells, and endogenous TNF-α-mediated apoptosis in Kym-1 cells (7). All these TWEAK-sensitive tumor cell lines expressed Fn14, as estimated by cell surface staining with ITEM-1 mAb (Fig. 4A). We also examined the expression of Fn14 on several TWEAK-resistant tumor cell lines. Although lymphoma cell lines such as Jurkat (Fig. 4A) did not express Fn14, some nonhemopoietic tumor cell lines such as SK-Hep-1 (Fig. 4A) and Hep3B (not shown) hepatomas and G361 and A375 melanomas (not shown) expressed substantial levels of Fn14. This suggested that the TWEAK sensitivity of tumor cells is not solely determined by the cell surface expression of Fn14.

We next examined whether soluble or immobilized ITEM-1 could induce cell death in these TWEAK-sensitive tumor cell lines (Fig. 4, B and C). Both soluble and immobilized ITEM-1 efficiently induced cell death in HT-29 cells, which was further enhanced by z-VAD-fmk. The dead HT-29 cells in the absence or presence of z-VAD-fmk exhibited apoptotic or necrotic morphology, respectively (data not shown). The necrotic death in the presence of z-VAD-fmk was inhibited by CA074 Me (Fig. 4, B and C), but not BHA (data not shown). Consistently, intracellular ROI level was not increased in HT-29 cells (data not shown). This suggested that the contribution of ROI to Fn14-mediated necrosis was cell type dependent. In HSC3 cells, especially immobilized ITEM-1 strongly induced caspase-dependent cell death, which was abrogated by z-VAD-fmk. In Kym-1 cells, especially immobilized ITEM-1 strongly induced cell death, which was mostly abrogated by anti-TNF-α mAb. All modes of the ITEM-1-induced cell death in these cell lines were just reminiscent of the TWEAK-induced cell death we observed in the previous study (7). In contrast, TWEAK-resistant Jurkat and SK-Hep-1 cells were also resistant to ITEM-1. These results indicated that ligation of Fn14 could induce caspase-dependent apoptosis, cathepsin B-dependent necrosis, or endogenous TNF-α-mediated cell death in particular cellular contexts, just like TWEAK stimulation.

We further examined whether the TWEAK-induced cell death in these tumor cell lines was solely mediated by Fn14. For this purpose, we used ITEM-2, which acted agonistic against hFn14/L5178Y cells (Fig. 3B), but could inhibit the binding of CD8-TWEAK to hFn14/L5178Y cells most efficiently (data not shown). Similar blocking effects of agonistic anti-death receptor mAbs on

FIGURE 3. Characterization of anti-hFn14 mAbs. A, Cell surface staining of Fn14 transfectants. L5178Y and hFn14/L5178Y cells were stained with biotinylated ITEM-1, ITEM-2, ITEM-3, or ITEM-4, followed by PE-labeled avidin (open histograms). Filled histograms indicate background staining with biotinylated control mAb plus PE-labeled avidin. B, Cytotoxic activity against Fn14 transfectants. L5178Y and hFn14/L5178Y cells were cultured with the indicated doses of soluble anti-Fn14 mAbs in the presence or absence of z-VAD-fmk (10 μM) for 48 h. Viability was measured by the WST assay. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.
ligand binding have been observed for Fas-Fas ligand or DR4-TRAIL interaction (30, 31). As shown in Fig. 5,
the binding of CD8-TWEAK to HT-29, HSC3, and Kym-1 cells was almost completely blocked by the preincubation with ITEM-2. Moreover, soluble ITEM-2 alone exhibited little cytotoxicity against these cell lines and rather completely blocked the CD8-TWEAK-induced cell death in these cell lines (Fig. 5B), which seemed to result from a weaker agonistic effect of ITEM-2 than CD8-TWEAK against these cell lines. These results suggested that Fn14 was solely responsible for the TWEAK-induced cell death in these cell lines.

In this study, we demonstrated that a single TWEAK receptor (Fn14) could mediate the multiple modes of TWEAK-induced tumor cell death in different cellular contexts. In contrast, Wiley et al. (13) have reported that Fn14 mediated proliferation in endothelial cells. Consistently, we also observed CD8-TWEAK or ITEM-1 could induce the proliferation of HUVEC (Fig. 1B and unpublished results). However, the signaling pathways leading to the multiple modes of cell death and proliferation remain unknown. Because Fn14 has been reported to lack a DD, but contain a TRAF binding domain, which interacts with TRAF1, 2, and 3 (13), it is possible that TRAFs and TRAF-binding proteins such as receptor-interacting protein (RIP) and TNFR-associated DD (32) may regulate the multiple pathways. It is well known that DD-containing TNFR1 and DD-lacking TNFR2 can also mediate multiple pathways leading to cell activation or death in a cell-dependent manner. Recently, it has been reported that TNFR2 signaling
leading to cell death or proliferation of T cells was dramatically affected by intracellular RIP levels (33). In this respect, Fas-mediated proliferation or death may be also determined by RIP expression and function of Fn14 in physiological and pathological conditions.

In this study, we also revealed that the inhibition of caspases by z-VAD-fmk sensitized hFn14/L5178Y and HT-29 cells to Fas-mediated necrotic death. However, the mechanisms for the negative regulation of necrotic death by caspases remain unknown. Recently, Holler et al. (34) have reported that Fas ligand, TRAIL, and HSC3 cells.

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