Apoptosis by Leukemia Cell-targeted Diphtheria Toxin Occurs via Receptor-independent Activation of Fas-associated Death Domain Protein

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ABSTRACT

Purpose: We examined the mechanism of action of a targeted fusion toxin consisting of diphtheria toxin fused to granulocyte macrophage colony stimulating factor (GMCSF) (DT\textsubscript{388}GMCSF), which was designed to selectively kill acute myeloid leukemia cells.

Experimental design and Results: U937 cells treated with DT\textsubscript{388}GMCSF underwent apoptosis as shown by chromatin degradation and cellular and nuclear fragmentation. This apoptosis was prevented by a general caspase inhibitor. DT\textsubscript{388}GMCSF treatment resulted in activation of the initiator caspases 8 and 9 and effector caspases. A selective caspase 8 inhibitor prevented activation of caspase 9, whereas a selective caspase 9 inhibitor did not prevent activation of caspase 8, indicating that caspase 8 activation is the proximal event in DT\textsubscript{388}GMCSF-induced apoptosis. Caspase 8 was activated through a Fas-associated death domain protein (FADD)-dependent mechanism as demonstrated by inhibition of DT\textsubscript{388}GMCSF-induced apoptosis on expression of a dominant negative FADD molecule. However, unlike most FADD-dependent apoptosis, this pathway may not involve death receptors, including Fas, tumor necrosis factor receptor 1, or tumor necrosis factor-related apoptosis-inducing ligand receptors, because inhibitors of the receptors did not prevent DT\textsubscript{388}GMCSF-induced apoptosis.

Conclusions: These data indicate that targeted toxins induce apoptosis by activating components of the death receptor pathway in a receptor-independent manner.

INTRODUCTION

In 2002, AML\textsuperscript{3} is estimated to be diagnosed in 10,600 people and cause 7,400 deaths in the United States (1). Standard therapy involves the use of 1-\beta-D-arabinofuranosylcytosine in combination with topoisomerase II inhibitors, such as etoposide. Most patients achieve remission with these drugs; however, they rapidly relapse, and only ~20% survive for >3 years (2). The dismal prognosis for patients who are diagnosed with AML underscores the need to develop novel therapies for this disease. One such therapy is a targeted toxin consisting of DT fused to GMCSF (DT\textsubscript{388}GMCSF), which shows promise in initial clinical trials (3).

Targeted toxins offer an exciting approach that may provide novel therapies for AML and other cancers (4, 5). Importantly, this approach may be applicable to tumor cells that are resistant to chemotherapy or radiation (6). The idea is straightforward. A targeting protein that recognizes and binds to tumor cells is attached to a toxic peptide. This protein enters the tumor cells, and the toxin kills the cell. Different kinds of targeting protein can be used, such as antibodies that recognize tumor cell-specific epitopes or ligands that bind to cell surface receptors on tumor cells (4). Toxins are derived from bacterial pathogens (e.g., DT or PE) or plants (e.g., ricin). These toxins block protein synthesis via different mechanisms. Ricin cleaves RNase to disrupt the ribosome, whereas DT and PE ADP-ribosylate elongation factor 2 to prevent the initiation of protein synthesis. This distinction is important because inhibitors that prevent DT- and PE-induced apoptosis do not block ricin-induced apoptosis, indicating that the mechanism of apoptosis induction by DT or PE and ricin is different (7). This suggests that apoptosis induction is not simply a consequence of protein synthesis inhibition but is instead dependent on the mechanism of action of the toxin. Because the toxin can only enter cells with receptors that recognize the targeting protein, only these cells are killed. The hope is that this selectivity will reduce side effects while maximizing tumor cell killing.

Despite the increasing development and use of targeted toxins (8), there is little known about how they kill tumor cells. DT fused to GMCSF induces apoptosis in AML cells from chemotherapy-resistant AML cell lines and primary leukemic cells from patients who are refractory to standard treatments (6). PE-fused immunotoxins can activate caspase 3-like activities

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3 The abbreviations used are: AML, acute myeloid leukemia; DT, diphtheria toxin; PE, pseudomonas exotoxin; FADD, Fas-associated death domain; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; YFP, yellow fluorescent protein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FADD-DN, Fas-associated death domain dominant negative; GMCSF, granulocyte macrophage colony stimulating factor.
that cleave known caspase substrates (9, 10). Furthermore, PE-immunotoxin-induced apoptosis was inhibited, and cell viability increased by treatment of T-cell leukemia cells with a general caspase inhibitor, z-VAD.fmk (10). In addition, a serine protease inhibitor has been reported to block toxin-induced apoptosis (11). These data suggest that caspase activation is an important aspect of targeted toxin-induced cell death; however, there is very little information regarding the mechanism that targeted toxins use to activate caspases.

Two main pathways leading to caspase activation and apoptosis have been described. The “extrinsic pathway” is activated by ligand-bound death receptors of the TNFR superfamily (12) through a mechanism that involves the adapter protein FADD, which activates the initiator caspase 8. Diverse stress pathways cause release of mitochondrial proteins to activate the other apoptosis pathway: the intrinsic pathway (13). This leads to activation of the initiator caspase 9. The extrinsic pathway can stimulate the intrinsic pathway through caspase 8 cleavage of Bid, which then translocates to mitochondria, causing release of mitochondrial proteins and activation of caspase 9 (14). This mechanism is thought to amplify the apoptotic signal.

In this study, we show that DT388-GMCSF kills U937 cells by inducing caspase-dependent apoptosis. The mechanism of caspase activation involves components of the extrinsic pathway, including FADD and caspase 8, but is independent of Fas, TNFR1, and TRAIL receptors. These data suggest that targeted DT uses a receptor-independent mechanism to activate FADD and caspase 8.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). DT388-GMCSF was prepared as described previously (15). Where indicated, DT388-GMCSF was added to the cell culture media at a concentration of 1.5 μg/ml. Antibodies against caspases were obtained from Cell Signaling (Beverly, MA). Caspase inhibitors were obtained from Calbiochem (La Jolla, CA) and used as recommended by the supplier. U937 cells were obtained from American Type Culture Collection and maintained as recommended by the supplier. Inhibitory antibodies for Fas (Chemicon International, Inc., Temecula, CA), TNFRI (R&D Systems, Inc., Minneapolis, MN), and TRAIL (R&D Systems) were used at 10 μg/ml. In control experiments, these receptors were activated using either an activating anti-Fas antibody (BD PharMingen, San Diego, CA) at 2 μg/ml, recombinant TNFα (R&D Systems) at 20 ng/ml in the presence of cycloheximide (10 mg/ml), or recombinant His-tagged TRAIL (R&D Systems) at 40 ng/ml with the addition of a cross-linking anti-His antibody at 10 μg/ml and actinomycin D (1 mg/ml).

Apoptosis Assays. Cells were harvested 7 h after treatment with or without DT388-GMCSF; total DNA was prepared by RNease A and proteinase K digestion and separated on agarose gels for analysis of DNA laddering. For analysis of nuclear morphology, cells were stained with Hoescht 33258 as described previously (16); cells were deposited onto slides using a cytopsin and scored for apoptotic nuclei by fluorescence microscopy.

RESULTS

DT388-GMCSF Induces AML Cell Apoptosis. Previous reports suggest that diphtheria fusion toxins, including DT388-GMCSF, can induce apoptosis (17). Before determining the mechanism by which this is achieved, we therefore first tested whether U937 cells displayed typical features of apoptosis when treated with the drug. One feature that is commonly associated with apoptosis is digestion of chromatin into a nucleosomal ladder. Fig. 1A shows that DT388-GMCSF caused DNA laddering that was prevented by treatment with zVAD.fmk, which inhibits most caspases (18). Other characteristics of
apoptosis are nuclear condensation and fragmentation. Fig. 1B shows that DT388-GMCSF treatment resulted in such typical apoptotic nuclear morphology. These data suggest that DT388-GMCSF causes caspase-dependent apoptosis in AML cells.

**DT388-GMCSF Activates Caspases 8 and 9 Along with Effector Caspases.** There are two well-characterized pathways by which caspases are activated. These pathways can be discriminated based on the initiator caspase that starts the caspase cascade. In the extrinsic death receptor pathway, the most common initiator caspase is caspase 8. Conversely, the intrinsic mitochondrial pathway involves activation of the initiator caspase 9. We therefore assayed activation of caspases 8 and 9 in response to DT388-GMCSF. Fig. 2, Lane 3 shows that the drug activated both initiator caspases as demonstrated by the appearance of the cleaved, active forms of the enzymes. As expected, caspase activation was blocked by zVAD.fmk (Fig. 2, Lane 4). In addition, we found that the effector caspase, caspase 3, was activated by DT388-GMCSF (Fig. 4B), and a hallmark caspase substrate, poly(ADP-ribose) polymerase, was cleaved (data not shown). These data indicate that caspases are activated but do not tell us whether the extrinsic or intrinsic pathway is activated first.

**Caspase 8 Activity Is Required for Caspase 9 Activation.** Caspase 8 activation can lead to caspase 9 activation through cleavage of Bid (14). This results in amplification of the apoptosis signal. Conversely, there are also examples where caspase 9 is activated first, and caspase 8 activation occurs as a later event (19). We tested whether caspase 8 activity was upstream or downstream of caspase 9 activity by treating cells with DT388-GMCSF in the presence of a caspase 8-specific inhibitor (IETD-CHO) or a caspase 9-specific inhibitor (LEHD-CHO). If caspase 8 is upstream of caspase 9, the caspase 8 inhibitor should prevent caspase 9 activation, but the caspase 9 inhibitor should not prevent caspase 8 activation. Fig. 2, Lanes 5 and 6 show that this was the case. Thus, DT388-GMCSF activates caspase 8, which then activates the intrinsic pathway to lead to caspase 9 activation.

**Dominant Negative FADD Prevents DT388-GMCSF-induced Apoptosis.** Caspase 8 is usually activated by death receptors through FADD. This activity can be blocked by expressing a FADD-DN that retains the death domain that binds to the receptors but does not contain a death effector domain to bind to caspase 8. We therefore tested if FADD-DN could prevent DT388-GMCSF-induced apoptosis. U937 cells were transfected with a YFP control or YFP-tagged FADD-DN and treated with DT388-GMCSF (DT-GM) as indicated. Fluorescent cells were identified, and each cell was scored for apoptotic nuclei after DNA stain. DT388-GMCSF induced apoptosis in the YFP-expressing cells but not in the cells expressing YFP-FADD-DN. Data shown are mean ± SE from three separate experiments using different preparations of cells and DNA molecules.

**DISCUSSION**

Targeted toxins, such as DT388-GMCSF, are becoming widely developed, and several such agents are being tested against different kinds of cancer (4, 8, 20). At least in part, these drugs work by inducing apoptosis in their target cells (6, 11). However, there is still little understanding of how these agents induce apoptosis. Furthermore, the available evidence suggests...
that the mechanisms of apoptosis may be different for different kinds of toxin (21) and either downstream or unrelated to toxin-induced protein synthesis inhibition (7). These data underscore the importance of understanding how targeted toxins induce apoptosis, because if different toxins induce apoptosis by different mechanisms, they may also differ in their ability to synergize with other agents or become sensitive to resistance mechanisms. Because DT$_{388}$-GMCSF is the first targeted toxin to enter clinical trials for treatment of AML (3), we focused our studies on this agent.

Our experiments indicate that DT$_{388}$-GMCSF kills myeloid leukemia cells by inducing caspase-dependent apoptosis. This apoptosis involves activation of caspase 8 through a FADD-dependent mechanism, but the mechanism does not appear to involve the known death receptors, including TNFR1, Fas, and TRAIL receptors. After caspase 8 is activated, the intrinsic pathway leading to activation of caspase 9 can be stimulated, which likely results in amplification of the apoptotic signal. Thus, DT$_{388}$-GMCSF-induced apoptosis is an example of a death receptor-independent mechanism of activating components of the extrinsic apoptosis pathway. There are a few other cases where this pathway can be activated to lead to caspase 8 activation but through a receptor-independent mechanism. The best understood example is neuronal apoptosis induced by the expanded polyglutamine-containing peptide that causes Huntington disease. This is caspase 8 and FADD dependent (22) but receptor independent. In Huntington disease, a protein called Hip-1, which binds to the polyglutamine peptide, recruits a binding protein called Hippi. Hippi also binds to caspase 8, and it is thought that this leads to receptor-independent caspase activation and apoptosis (23).

How might DT$_{388}$-GMCSF activate caspase 8? One possibility is that DT$_{388}$-GMCSF treatment alters the activity of a protein that functions like Hippi to recruit caspase 8. An alternate mechanism is that DT$_{388}$-GMCSF inhibits an inhibitor of caspase 8 activation, leading to an increase in the basal level of caspase activity in DT$_{388}$-GMCSF-treated cells. One attractive candidate is c-FLIP, an endogenous inhibitor that competes with caspase 8 for binding to FADD, thus preventing activation of apoptosis (24, 25). Down-regulation of c-FLIP by metabolic inhibitors can alter signaling responses to death ligands (26) and sensitize cells to Fas, TNF, or TRAIL-induced apoptosis (25). However, if down-regulation of c-FLIP was responsible for DT$_{388}$-GMCSF-induced apoptosis by sensitizing death receptors, this should still require death ligand signaling. In this case, we should have found that the inhibitory antibodies, which block FasL, TNF$\alpha$, or TRAIL-induced apoptosis, would have had at least some effect on DT$_{388}$-GMCSF’s ability to induce apoptosis. Because this was not the case, we suggest that down-regulation of c-FLIP is likely not responsible for apoptosis.

Our identification of components of the extrinsic apoptosis pathway as being responsible for induction of apoptosis by
DT_{388}-GMCSF provides a basis for future studies to further characterize this mechanism in U937 and other cell types. Chemotherapy drugs synergize with DT_{388}-GMCSF to kill cells more effectively than either agent alone (27, 28). Studies to further understand the mechanism by which the extrinsic apoptosis pathway machinery is activated by DT_{388}-GMCSF are under way. These studies may provide a mechanistic basis to understand why such synergy occurs and test resistance mechanisms that could explain why some patients do not respond to the drug (3).

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**REFERENCES**

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