Induction of Apoptosis in Multidrug-resistant and Radiation-resistant Acute Myeloid Leukemia Cells by a Recombinant Fusion Toxin Directed against the Human Granulocyte Macrophage Colony-stimulating Factor Receptor

John P. Perentesis, Kevin G. Waddick, Anne E. Bendel, Yu Shao, Berta E. Warman, Mridula Chandan-Langlie, and Fatih M. Uckun

Biotherapy Institute [J. P. P., K. G. W., Y. S., F. M. U.] and Departments of Pediatrics [J. P. P., A. E. B., Y. S., B. E. W., F. M. U.], Therapeutic Radiology-Radiation Oncology [K. G. W., Y. S., F. M. U.], Biochemistry [J. P. P.], and Pharmacology [F. M. U.], University of Minnesota Medical School and Academic Health Center, Minneapolis, Minnesota 55455, and Hughes Institute, St. Paul, Minnesota 55113 [M. C.-L.]

ABSTRACT

Multianti chemotherapy regimens fail to cure more than one-half of the patients with acute myeloid leukemia (AML) because of the emergence of dominant multidrug-resistant subclones of leukemia cells. We have developed a recombinant diphtheria toxin-human granulocyte macrophage colony-stimulating factor chimeric fusion protein (DT-GMCSF) that specifically targets GMCSF receptor-positive AML cells. This novel biotherapeutic agent induced rapid apoptotic cell death of chemotherapy-resistant AML cell lines and primary leukemia cells from treatment-refractory AML patients. Our results suggest that DT-GMCSF may be useful in the treatment of AML patients whose leukemia has recurred and developed resistance to contemporary chemotherapy programs.

INTRODUCTION

AML is the most common form of acute leukemia in adults and the second most frequent leukemia in children (1, 2). Multiagent chemotherapy regimens fail to cure more than one-half of patients with AML because of the emergence of dominant multidrug-resistant subclones of leukemia cells (3). Myeloblastic chemotherapy and supralethal radiochemotherapy followed by allogeneic or autologous bone marrow transplantation have been used in an attempt to overcome drug resistance in AML. These regimens are associated with considerable morbidity and mortality, frequently fail to eradicate multidrug-resistant or radiation-resistant leukemia cells, and have effected only modest improvements in the overall survival of AML patients, underscoring the need for rational drug design-based therapies for AML (4–6).

Native DT is a 535-residue protein secreted by Corynebacterium diphtheriae and one of the most toxic substances found in nature (7). A single DT molecule entering a human cell can induce catalytic inactivation of protein synthesis, leading to rapid cell death (7). DT inhibits protein synthesis by catalyzing the ADP-ribosylation and inactivation of elongation factor 2, an essential protein synthesis cofactor, at a highly conserved posttranslationally modified histidine residue known as diphthamide (8). Its profound toxicity is a result of the catalytic nature of its mechanism of action, as well as the ubiquitous expression of its receptor, a heparin-binding EGF-like precursor, on human cells (9). Biochemical (10) and genetic (11–13) analyses of DT have provided tremendous insights into its structure-function relationships. These investigations have also guided the design of novel recombinant therapeutic agents that were constructed by genetic deletion of the receptor binding domain of the toxin and its replacement with growth factors, serving to effectively redirect the toxin to growth factor receptors found on malignant cells (14–18). In addition, recent X-ray crystallographic analyses (19, 20) have expanded upon these investigations to identify three functionally distinct domains within the DT structure: (a) an amino-terminal catalytic domain ("C" domain) that contains the ADP-ribosyltransferase active site; (b) a transmembrane domain ("T" domain) found in the middle of the protein and facilitating C domain translocation across membranes; and (c) a carboxyl-terminal receptor binding domain ("R" domain) that mediates binding and leads to receptor-mediated endocytosis. These structural studies have also revealed that DT amino acid residues 380–386, located in a small loop separating the receptor binding domain from the catalytic and transmembrane domains, allow the entire M, 15,000 receptor binding domain to flexibly rotate as a unit by 180°, with atomic movement of up to 15,000 residues.
65 Å. Separate studies have elucidated the crystal structure of GMCSF (21, 22), demonstrated that it is a member of the four-helix bundle family of cytokines (23), and defined critical domains that are essential for high-affinity binding to its receptor (GMCSF-R).

We have built upon the investigations of the molecular and structural biology of DT and GMCSF to create a novel fusion toxin, DT 
\text{GMCSF}, that preserves the catalytic and transmembrane domains of DT but replaces the native toxin receptor binding domain with human GMCSF and includes a sterically neutral peptide linker separating the toxin and growth factor domains at the site of the flexible peptide loop at residues 380–386. Here, we demonstrate that DT 
\text{GMCSF} is able to redirect the protein synthesis inhibitory action of DT to multidrug-resistant as well as radiation-resistant AML cells, resulting in apoptotic cell death. The mechanism of action of DT 
\text{GMCSF} is different from those of other antileukemic drugs used in contemporary AML therapy programs and may allow us to overcome drug and radiation resistance in AML.

**MATERIALS AND METHODS**

**DT 
\text{GMCSF Fusion Toxin.}** The engineering and production of DT 
\text{GMCSF fusion toxin in highly purified form were described in detail in a previous report from our laboratory (24). In brief, DT 
\text{GMCSF is a 521-amino acid residue chimeric protein containing a predicted amino-terminal methionine residue, followed by amino acid residues 1–385 of DT, a Ser-(Gly)4-Ser-Met linker peptide, and mature human GMCSF. This fusion toxin preserves the portions of DT, including the lethal catalytic ADP-ribose transferase domain (C domain) and the contiguous proximal portion of the toxin that is associated with translocation across cellular membranes (T domain). The native receptor binding domain of DT was completely deleted in the construction of the DT 
\text{GMCSF fusion toxin. The short Ser-(Gly)4-Ser-Met linker peptide was inserted in the fusion toxin at the natural DT receptor "hinge site" to separate the DT and GMCSF moieties and insure that the NH2-terminal helices of GMCSF would be accessible for high-affinity receptor binding. DT 
\text{GMCSF was expressed with high-efficiency fermentation methods in Escherichia coli} and purified through sequential anti-DT immunoaffinity and mono-Q high-pressure liquid chromatographic methods, followed by endotoxin removal (24, 25).**

**Cell Lines and Culture Conditions.** GMCSF-R-bearing human leukemia cell lines included the human acute promyelocytic leukemia cell line HL-60 (26) and the GMCSF-R-negative control leukemia cell line K562 (27) obtained from the American Type Culture Collection (Rockville, MD). Multidrug-resistant subclones of HL-60 cells, including HL-60/VCR cells (28), which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells (29, 30), which express a MRP-associated MDR phenotype, were the gift of Dr. M. Center (Kansas State University, Manhattan, KS). HL-60 cells were maintained in IMDM, 20% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. K562 cells were maintained in RPMI, 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Primary leukemia cells were obtained from the previously cryopreserved AML bone marrow samples of therapy-refractory patients stored in the liquid nitrogen tanks of the Children’s Cancer Group Cell Bank at the University of Minnesota.

**GMCSF Displacement Assay.** Ligand displacement assays were performed as described previously (31). Radiolabeled \text{125-I-GMCSF} (Dupont-NEN) at a concentration of 40 pm and unlabeled DT 
\text{GMCSF or recombinant human GMCSF} at increasing concentrations (1 pm to 100 nm) were incubated at 4°C with 4 × 106 HL-60 myeloid leukemia cells in 400 μl of IMDM binding buffer. After a 2-h incubation, cells were transferred into 0.75 ml of an ice-cold mixture of 75% FBS in binding buffer, and the cells were then collected by centrifugation. A gamma counter was used to measure radioactivity in the supernatants and pellets.

**Protein Synthesis Inhibition Assays.** For protein synthesis inhibition assays, 1 × 10⁶ cells/well were seeded into 96-well sterile MultiScreen-HV plates (Millipore Corp.) containing leucine-free RPMI followed by the addition of DT 
\text{GMCSF, native recombinant human GMCSF (Immunex), or PBS, and incubated at 37°C for 24 h. RPMI plus \text{[3H]leucine}} (l-[3,4,5-3H]; DuPont-NEN) was added to a final concentration of 1 μCi/well with a 2-h pulse incubation. Using a MultiScreen system vacuum manifold, the cells were washed twice with PBS and lysed with sterile water, and the insoluble protein was precipitated in 20% TCA. The insoluble protein was washed three times with 10% TCA, and radioisotope incorporation into protein as collected on the MultiScreen-HV plate filter was measured in a Beckman LS7000 scintillation counter after a 30-min incubation in 0.42% sodium hypochlorite. Chemiluminescence sodium hypochlorite controls were prepared daily.

**Apoptosis Assays.** To detect apoptotic changes, cells were harvested 24 h after continuous exposure to the DT 
\text{GMCSF fusion toxin, and DNA from supernatants of Triton X-100 lysates was prepared for analysis of fragmentation, as described previously in detail (32, 33). DNA was separated by electrophoresis through a 1% agarose gel, and the DNA fragments were visualized by UV light after being stained with ethidium bromide. Controls included DNA from PBS-treated cells cultured for 24 h, DNA from cells treated with 1000 ng/ml GMCSF for 24 h, DNA from cells irradiated with 2 Gy γ-rays 24 h prior to harvest, and DNA from cells preincubated for 2 h with 3000 ng/ml native recombinant GMCSF prior to treatment with 100 ng/ml DT 
\text{GMCSF for 24 h}.**

**RESULTS**

DT 
\text{GMCSF-induced Apoptosis in GMCSF Receptor-positive Human AML Cells.} DT 
\text{GMCSF contains both the catalytic and transmembrane translocation domains of DT linked via a short, sterically neutral peptide incorporated as a spacer to GMCSF, replacing the DT receptor binding domain (Fig. 1A). HL-60 is a p53-deficient myeloid leukemia cell line expressing 77 ± 12 high-affinity GMCSF-R/cell with a Kd of 34.8 ± 3.8 pm (24). As shown in Fig. 1B, DT 
\text{GMCSF exhibited high-affinity binding to the GMCSF-R on HL-60 cells and was able to displace \text{125-I-GMCSF from HL-60 myeloid leukemia cells with an EC₅₀ value (i.e., concentration that effected 50% displacement of \text{125-I-GMCSF from the GMCSF-R}} of 0.199 nm. DT 
\text{GMCSF effectively inhibited}}
protein synthesis in HL-60 cells with an IC50 of approximately 0.5 ng/ml (~9 pm; Fig. 1C). Native human GMCSF did not inhibit protein synthesis in HL-60 cells, and the protein synthesis-inhibitory activity of DTc-GMCSF could be abolished by preincubation of cells with excess native human GMCSF, which competes for binding to the GMCSF-R. In contrast, protein synthesis in the erythroleukemia cell line K562, which does not express the high-affinity GMCSF-R (34), was not affected by DTc-GMCSF concentrations as high as 1000 ng/ml (~17 nM). Thus, the observed inhibition of protein synthesis in DTc-GMCSF-treated HL-60 cells was mediated through specific binding of this fusion toxin to the high-affinity GMCSF-R.

Because the final pathway for cell death after intoxication with DTc-GMCSF is unknown, we investigated whether the inhibition of protein synthesis effected by the DTc-GMCSF-specific ADP-ribosylation of the diphthamide site of elongation factor 2 could subsequently trigger apoptosis in GMCSF-R-positive human leukemia cells. The failure of normal apoptosis pathways or resistance to chemotherapy-induced apoptosis is an important mechanism underpinning the biology of chemotherapy-refractory leukemias. Several studies have demonstrated that inhibitors of protein synthesis, including DT and cycloheximide, activate apoptotic cell death programs in a variety of leukemia cell lines (35, 36). We tested the cytotoxicity of DTc-GMCSF against HL-60 cells deficient in p53 because mutation of the p53 gene and loss of its functional tumor suppressor activity is frequently observed in aggressive malignancies and is associated with a failure of the induction of apoptotic cell death and a poor response to conventional therapies that damage DNA (37, 38). Within 4 h of exposure to 100 ng/ml of DTc-GMCSF, approximately 50–75% of HL-60 cells showed morphological changes consistent with extensive apoptotic damage, including pronounced shrinkage of the cells, nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebbing (Fig. 2). In contrast, native human GMCSF did not induce apoptosis in HL-60 cells (Fig. 2B). A 24-h exposure to 100 or 1000 ng/ml DTc-GMCSF was more effective than a 4-h exposure; after 24 h of treatment, very few cells remained detectable, and virtually all of these cells showed signs of extensive damage consistent with apoptosis (Fig. 2). A few isolated cells showed distinctive features of necrosis, including swelling of the nucleus and cytoplasm and loss of nuclear and cytoplasmic basophilia (Fig. 2E).

Examination of DNA from DTc-GMCSF-treated (but not GMCSF-treated) HL-60 cells on agarose gels revealed a dose-dependent and ladder-like fragmentation pattern consistent with endonucleolytic cleavage of the DNA into oligonucleosome-length fragments during apoptotic cell death (Fig. 3). DTc-GMCSF did not cause DNA fragmentation in the GMCSF-R-negative NALM-6 pre-B lineage leukemia cell line, and preincubation of the HL-60 cells with excess native GMCSF was able to prevent DNA fragmentation (Fig. 3A and B). Thus, DTc-GMCSF-induced apoptosis was mediated by the GMCSF-R-specific binding of the fusion toxin to leukemia cells. DTc-GMCSF-induced Apoptosis in Multidrug-resistant and Radiation-resistant Human AML Cells. We next studied the antileukemic activity of DTc-GMCSF against HL-60/VCR cells, which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells, which express a MRP-
associated MDR phenotype. On agarose gels, DNA from DTcGMCSF-treated HL-60/VCR and HL-60/ADR myeloid leukemia cells exhibited a ladder-like fragmentation pattern consistent with apoptosis (Fig. 3C). Thus, MDR due to over-expression of P-glycoprotein or MRP does not protect GMCSF-R-positive AML cells from DTcGMCSF-induced apoptosis.

We also studied the ability of DTcGMCSF to induce apoptosis in primary leukemic cells from four patients with chemotherapy-refractory relapsed AML. In one case (case 1), DTcGMCSF was as effective as 2 Gy γ-rays in inducing apoptosis, and in three additional cases (cases 2, 3, and 4), DTcGMCSF induced DNA fragmentation, whereas 2 Gy γ-rays

**Fig. 2** Morphological features of DTcGMCSF-treated leukemia cells undergoing apoptosis. After treatment with 100 ng/ml DTcGMCSF for 4 or 24 h, HL-60 cells were examined on Wright-Giemsa-stained cytospin slides for morphological changes characteristic of apoptosis, including nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebs. Apoptotic cells (AP) and apoptotic bodies (AB), necrotic cells (NC) as well as morphologically healthy cells (NL), are indicated by arrows. Control cells were treated for 24 h with PBS (CON) or 100 ng/ml native recombinant GMCSF.
Fig. 3 Analysis of DT₀GMCSF dose-dependent induction of internucleosomal DNA fragmentation in GMCSF-R-positive leukemia cells. Cells were harvested 24 h after continuous exposure to the DT₀GMCSF fusion toxin, and DNA from supernatants of Triton X-100 lysates was prepared for analysis of fragmentation. DNA was separated by electrophoresis through a 1% agarose gel, and the DNA fragments were visualized by UV light after staining with ethidium bromide. Controls included DNA from PBS-treated cells cultured for 24 h (CON), DNA from cells treated with 1000 ng/ml GMCSF for 24 h (GMCSF), and DNA from cells preincubated for 2 h with 3000 ng/ml GMCSF prior to treatment with 100 ng/ml DT₀GMCSF for 24 h (GMCSF → DT₀GMCSF). A, p53-deficient HL-60 myeloid leukemia cells bearing high-affinity GMCSF-R. B, GMCSF-R-negative NALM-6 pre-B leukemia cells. C, multidrug-resistant HL-60/ADR leukemia cells expressing high levels of Pgp and HL-60/ADR leukemia cells expressing MRP treated with DT₀GMCSF. Molecular size markers (in bp) are indicated at the left in each panel.
Fig. 4  DT<sub>3</sub>GMCSF induces apoptosis in primary leukemic cells from relapsed AML patients. Bone marrow-derived leukemic cells from 4 therapy-refractory AML patients in relapse were harvested 24 h after continuous exposure to 100 ng/ml DT<sub>3</sub>GMCSF fusion toxin or PBS (CON), or irradiation with 2 Gy γ-rays, and DNA from Triton X-100 lysates was prepared for analysis of fragmentation. Molecular size markers (in bp) are indicated at the left of each panel.

DISCUSSION

The emergence of chemotherapy- and radiation-resistant clones of leukemic cells is the major obstacle to the cure of AML and is caused by several mechanisms. The multidrug resistance phenotype has been implicated as one such major cause for treatment failure in AML and results from overexpression of transmembrane transporter molecules exporting a variety of natural cytotoxic agents with different mechanisms of action (39, 40). The MDR phenotype in AML can be artificially produced by the overexpression of Pgp (also known as P-170; Refs. 41 and 42), and the MRP (29, 43, 44). Expression of Pgp in primary leukemic cells from AML patients has been associated with higher rates of chemotherapy remission induction failures, shorter relapse-free intervals, and increased rates of relapse (45–50). In addition, increased MRP expression has also been identified in the leukemic cells of a significant proportion of patients with relapsed AML (51, 52). Attempts to overcome drug resistance in AML by increasing chemotherapy regimen dose intensity, including myeloablative chemotherapy followed by allogeneic or autologous bone marrow transplantation, or by the use of agents that inhibit the activity of MDR-associated transporter molecules, have been associated with considerable morbidity and mortality, and have effected only modest improvements in the overall survival of AML patients (4–6). The utility of dose-intensive therapy in the management of therapy-resistant malignancies is severely limited because of the toxicities inflicted upon normal tissues.

Paralleling the contribution of the MDR phenotype to the poor survival of AML patients are the observations that clinically aggressive and drug-resistant forms of AML are associated with the autonomous growth of leukemic blasts related to the activation of hematopoietic growth factor-related proliferative pathways (53, 54). In particular, GMCSF and its receptor appear to play a prominent role in AML leukemogenesis, and large subsets of patients may possess leukemic cells with direct activation of GMCSF-related growth loops (55–57) or indirect activation of GMCSF growth loops caused by interleukin 1 or other cytokines (58–60). The role of GMCSF in the etiology and maintenance of AML is underscored by the observation that autonomous proliferation of AML blasts in culture can be abrogated in over 80% of patient samples by exposure to either a neutralizing anti-GMCSF antibody or an antisense oligonucleotide directed against the GMCSF transcript (61). Moreover, there is substantial evidence that the leukemic blasts from the majority of patients with AML express GMCSF-R (62–64), and GMCSF-specific binding can be demonstrated to leukemic cells from virtually all AML samples surveyed when refined binding studies are used (65). The observation that GMCSF and its receptor may function prominently in the etiology and maintenance of AML has prompted attempts to use it as a "priming agent" to stimulate the transit of leukemic cells into S phase and thereby increase sensitivity to cytotoxic chemotherapies (66–71). Although this approach is intriguing, it does not provide a vehicle to overcome important AML therapy resistance resulting from MDR-associated mechanisms or resistance to apoptotic cell death.
A novel strategy for tumor-specific biotherapy has been developed by using genetic engineering to target protein synthesis inhibiting toxins, including DT (14–19) and Pseudomonas exotoxin A (72, 73), to growth factor receptors found on neoplastic cells. Notably, it has been observed previously that Pseudomonas exotoxin A can effectively kill drug-resistant cancer cells that express the Pgp (74). We have used genetic engineering to create a novel fusion toxin, DT\textsubscript{5}GMCSF, to redirect the lethal action of DT to the high-affinity GMCSF-R found on AML cells. We postulated that DT\textsubscript{5}GMCSF would be an active agent against drug-resistant leukemia cells possessing the MDR phenotype or deficient in p53 expression because (a) it gains entry to target cells by a unique mechanism (GMCSF-R binding); (b) it kills cells by a mechanism of action (protein synthesis inhibition) that is distinct from other chemotherapeutic agents and radiation; and (c) it is not apparently similar in structure to other Pgp or MRP substrates. Our results presented in this report demonstrate that DT\textsubscript{5}GMCSF is able to overcome classical mechanisms of drug and radiation resistance, as well as circumvent the failure to engage apoptotic pathways. The recombiant fusion toxin DT\textsubscript{5}GMCSF may thus serve as an effective treatment for AML.

Resistance to the induction of the programmed cell death mechanism after malignant cells have incurred damage from chemotherapy or radiation is another important mechanism of treatment resistance in AML and other cancers (75). Known also by the descriptive term apoptosis, this specific cell death mechanism is characterized by distinct morphological and ultrastructural features and by endonuclease-mediated cleavage of DNA into oligonucleosome-length fragments (76–78). Ionizing radiation and chemotherapeutic drugs have been shown to inflict cellular damage that engage cellular mechanisms, likely including the interleukin-1\textbeta-converting enzyme protease pathways, that result in the apoptotic cell death of cancer cells (79–81). The p53 tumor suppressor gene appears to play a major role in induction of apoptosis (37, 38). Cancers with deficient function of p53 are thus postulated to be therapy resistant (82), and treatment resistance and relapse have been associated with inactivation of p53 and defective induction of apoptosis in immunocompromised mouse tumor models (83). Human hematological malignancies bearing p53 gene mutations exhibit \textit{in vitro} resistance to DNA-damaging agents, including chemotherapy and radiation (84), and are also associated with a poor response to therapy and short survival (85, 86). In our studies, treatment of p53-deficient HL-60 myeloid leukemia cells with DT\textsubscript{5}GMCSF resulted in the rapid and efficient induction of apoptosis, suggesting that the fusion toxin could potentially circumvent chemotherapy and radiation resistance resulting from p53-deficiency and failure of apoptosis. The DT\textsubscript{5}GMCSF-induced DNA fragmentation and apoptotic alterations in morphology in HL-60 leukemia cells were not associated with evidence of terminal maturation, nor were they associated with growth factor deprivation, and these cells are GMCSF independent. DT\textsubscript{5}GMCSF-induced DNA fragmentation was most likely a consequence of the DT catalytic domain-induced inhibition of protein synthesis because native recombinant GMCSF did not cause DNA fragmentation, and a variety of biochemical and genetic studies have demonstrated that the DT catalytic domain does not possess direct DNase activity (87–89). These observations suggest that, at least in some leukemias, the induction of apoptosis by DT\textsubscript{5}GMCSF-mediated inhibition of protein synthesis involves p53-independent pathways. The precise pathway of apoptosis induction is not yet defined, and we are examining whether cells with defects in the downstream effectors of apoptotic cell death (e.g., the interleukin-1\textbeta-converting enzyme protease pathways) remain susceptible to the cytotoxic consequences of treatment with DT\textsubscript{5}GMCSF.

We further postulated that DT\textsubscript{5}GMCSF would be an active agent against leukemia cells possessing the MDR phenotype because its structure is distinct from other P-glycoprotein or MRP substrates and it kills target cells by a unique mechanism of action. We found that overexpression of P-glycoprotein or MRP does not appear to cause a significant decrease in DT\textsubscript{5}GMCSF cytotoxicity in human myeloid leukemia cells and that DT\textsubscript{5}GMCSF efficiently induces apoptosis in highly drug-resistant cells. The prominent role of the MDR phenotype in causing treatment failure and ultimate mortality in AML suggests that DT\textsubscript{5}GMCSF may be useful to complement the activity of conventional antineoplastic agents in the treatment of AML.

REFERENCES


Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia cells by a recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/3/3/347

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.