Diphtheria toxin-murine granulocyte-macrophage colony-stimulating factor–induced hepatotoxicity is mediated by Kupffer cells

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Abstract

DT_{388}GMCSF, a fusion toxin composed of the NH_2-terminal region of diphtheria toxin (DT) fused to human granulocyte-macrophage colony-stimulating factor (GMCSF) has shown efficacy in the treatment of acute myeloid leukemia. However, the primary dose-limiting side effect is liver toxicity. We have reproduced liver toxicity in rats using the rodent cell-tropic DT-murine GMCSF (DT_{390}mGMCSF). Serum aspartate aminotransferase and alanine aminotransferase were elevated 15- and 4-fold, respectively, in DT_{390}mGMCSF-treated rats relative to controls. Histologic analysis revealed hepatocyte swelling; however, this did not lead to hepatic necrosis or overt histopathologic changes in the liver. Immunohistochemical staining showed apoptotic cells in the sinusoids, and depletion of cells expressing the monocyte/macrophage markers, ED1 and ED2, indicating that Kupffer cells (KC) are targets of DT_{390}mGMCSF. In contrast, sinusoidal endothelial cells seemed intact. *In vitro*, DT_{390}mGMCSF was directly cytotoxic to primary KC but not hepatocytes. Two related fusion toxins, DT_{388}GMCSF, which targets the human GMCSF receptor, and DT_{390}mIL-3, which targets the rodent IL-3 receptor, induced a less than 2-fold elevation in serum transaminases and did not deplete KC *in vivo*. In addition, DTU2mGMCSF, a modified form of DT_{390}mGMCSF with enhanced tumor cell specificity, was not hepatotoxic and was significantly less toxic to KC *in vivo* and *in vitro*. These results show that DT_{390}mGMCSF causes liver toxicity by targeting KC, and establish a model for studying how this leads to hepatocyte injury. Furthermore, alternative fusion toxins with potentially reduced hepatotoxicity are presented. [Mol Cancer Ther 2004;3(12):1681–9]

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Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults in the United States. With combination induction and consolidation therapy using cytarabine and topoisomerase II inhibitors, remissions could be achieved (1). However, due in large part to the persistence of leukemic blasts that are resistant to conventional DNA replication-targeted drugs, most patients ultimately relapse and die from the disease or complications of treatment (1). New therapeutics are urgently needed for the treatment of this disease.

Fusion toxins are a novel class of drugs designed to target leukemia cells specifically, by mechanisms other than damaging DNA or blocking cell division. These drugs are chimeras of protein synthesis-inhibiting toxins fused genetically to ligands specific for leukemia blasts (2). One such fusion toxin, DT_{388}GMCSF, is composed of the catalytic and translocation domains of diphtheria toxin (DT) fused to granulocyte-macrophage colony stimulating factor (GMCSF; refs. 3, 4). *In vitro*, DT_{388}GMCSF was significantly more cytotoxic to GMCSF receptor-positive AML blasts than to normal hematopoietic progenitors (5, 6). DT_{388}GMCSF showed clinical efficacy in producing partial and complete remissions in a phase I trial of patients with relapsed or refractory AML (7). However, liver damage was the predominant side effect that limited dose escalation. Understanding the mechanism of DT_{388}GMCSF-induced liver injury could lead to treatments designed to block liver toxicity while preserving antileukemic efficacy of the drug.

In toxin-sensitive cells, DT_{388}GMCSF binds to and is internalized by GMCSF receptors. Once in endosomes, DT is cleaved by furin, a ubiquitous serine protease (8). The A fragment of DT is then translocated to the cytosol where it catalyzes the ADP-ribosylation of elongation factor-2 (9), resulting in inhibition of protein synthesis and cell death (10). In the study presented here, we have utilized a rodent-tropic homologue of DT_{388}GMCSF, DT_{390}mGMCSF (11), to investigate the mechanism of liver toxicity in a rat model. We have furthermore compared the effects of DT_{390}mGMCSF with two different but related fusion toxins, DT_{390}mIL-3 (12), and DTU2mGMCSF. DT_{390}mIL-3 is a rodent-tropic homologue of DT_{388}IL-3, a chimera of DT

and IL-3, which is in early phase I clinical trials for the treatment of AML. Similarly, DTU2mGMCSF is a rodent-tropic homologue of DTU2GMCSF, a modified human GMCSF receptor-targeted fusion toxin. DTU2GMCSF is genetically identical to DT_{388}GMCSF except that the native furin cleavage site in DT has been replaced by a urokinase plasminogen activator (uPA) cleavage site (13). uPA is a secreted serine protease that on cleavage by plasmin, binds to the urokinase plasminogen activator receptor (uPAR). The active uPA/uPAR complex functions in the degradation of extracellular matrix (14). The uPA/uPAR system is overexpressed by many tumors including myeloid leukemias, whereas its expression on normal cells is restricted to certain physiologic processes such as wound healing and tissue remodeling (14, 15). Thus, DTU2GMCSF was designed as a dual specificity fusion toxin to target cells expressing both GMCSF receptor and an active uPA/uPAR system. We have recently reported that AML cell lines are highly sensitive to DTU2GMCSF *in vitro* (13); however, the *in vivo* effects of this fusion toxin have not been reported.

In an effort to begin to define the molecular mechanism by which DT_{388}GMCSF leads to liver injury, we sought to establish a model that would allow us to examine the susceptibility of liver cell populations to DT fusion toxins *in vivo* and *in vitro*. This report shows that like DT_{388}GMCSF in humans, DT_{390}mGMCSF induces liver injury in Sprague-Dawley rats. Furthermore, using our panel of modified DT-conjugated toxins, we show that the capacity of fusion toxins to damage the liver correlates directly with the susceptibility of Kupffer cells (KC) to their cytotoxic effects.

Materials and Methods

Toxins

The construction, expression, and purification of DT_{388}GMCSF (3), DT_{390}mGMCSF (11), and DT_{390}mIL-3 (12) have been reported previously. To construct DTU2mGMCSF, DTU2GMCSF expression plasmid pRKDTGM-U2 (13) was modified by replacement of the *Nde*I-*Eco*RI fragment coding for the human GMCSF with an *Nde*I-*Eco*RI fragment that encodes for the mouse GMCSF. The mouse GMCSF coding sequence was amplified by PCR using 5′ primer M1 (CCTCATATGGCACCCACCCGCTCACCCATC, the *Nde*I site is underlined) and 3′ primer M2 (CCGAATTCATTTTTGGACTGGTTTTTTGCA, the *Eco*RI site is underlined). A mouse GMCSF cDNA containing plasmid was used as a template. The resulting PCR product was digested with *Nde*I and *Eco*RI, and was then cloned into the *Nde*I-*Eco*RI sites of pRKDTGM-U2, yielding DTU2 mGMCSF expression plasmid, pRKDTmGM-U2, which was confirmed by DNA sequencing analysis. Ricin was purchased from Sigma-Aldrich (St. Louis, MO) and was stored and handled according to guidelines set by the Wake Forest University School of Medicine Office of Environmental Health and Safety.

Cell Lines

The J774A.1 macrophage cell line was obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. FBL-3 (12, 16) was grown in RPMI medium 1640 supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, glutamine, and 10 μg/mL gentamicin. Cells were maintained in their respective growth medium for *in vitro* cytotoxicity assays.

In vitro Cytotoxicity Assay

To measure cytotoxicity *in vitro*, tumor cells (1×10^4 per well) were incubated in triplicate in 96-well flat-bottomed dishes with serial dilutions of fusion toxins as indicated. After 48 hours at 37°C, 5% CO_2, 1 μCi/well [^{3}H]thymidine was added and the cells were incubated for an additional 18 hours. The cells were then harvested onto glass fiber filters mats using a Skatron cell harvester (Skatron Instruments, Lier, Norway) and the amount of radiolabeled thymidine incorporated into DNA was measured using a Betaplate liquid scintillation counter (Perkin-Elmer, Boston, MA). The percentage of maximal [^{3}H]thymidine incorporation was plotted versus the log of the toxin concentration, and nonlinear regression with a variable slope sigmoidal dose response curve was generated along with the EC_{50} using GraphPad Prism software. The EC_{50} was defined as the concentration of toxin that inhibited [^{3}H] thymidine incorporation by 50% as compared with control cells incubated in medium without toxin. Cytotoxicity assays were done at least thrice for each tumor cell line and representative experiments are shown.

In vitro Toxicity Studies

Female Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were used for all experiments. Rats were treated with 200 μg/kg fusion toxins. Animals were sacrificed after 12 to 18 hours by CO_2 inhalation and blood was collected from the inferior vena cava. Serum was analyzed for the presence of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by the clinical chemistry laboratory at Wake Forest University Medical Center. For histologic and immunostaining procedures, liver samples were fixed in 10% neutral buffered formalin and embedded in paraffin or snap-frozen in Tissue Tek optimum cutting temperature compound and stored at −80°C prior to preparing sections. For histopathologic evaluation, liver sections representative of each group of rats were stained with H&E. For transaminase data, statistical significance was analyzed by one-way ANOVA. *In vivo* experiments were done at least twice for each fusion toxin, and representative experiments are shown.

Electron Microscopy

Liver samples obtained from control and toxin-treated rats were fixed in 2.5% glutaraldehyde and processed by the electron microscopy laboratory at Wake Forest University School of Medicine using standard methods. Toluidine blue–stained, plastic-embedded thick sections were examined and photographed under light microscopy. Representative areas were further processed for thin sections and examination by transmission electron microscopy.

Immunohistochemistry to Detect Apoptotic Cells

Formalin-fixed paraffin sections were deparaffinized and incubated at 95°C for 1 hour in antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by a 20-minute cool-down at room temperature. All subsequent steps were done at room temperature. To block endogenous peroxidase, slides were incubated for 10 minutes in 0.3% H_2O_2. After washing thrice with water and thrice with PBS, the sections were blocked for 10 minutes with 10% normal goat serum in PBS. Blocker was drawn off with a pipette and replaced with rabbit anticleaved caspase 3 (Cell Signaling Technologies, Beverly, MA) diluted 1:100 in PBS + 1% normal goat serum. After 1 hour, slides were washed thrice with PBS and treated with goat anti-rabbit IgG-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA; 1:250 in PBS + 1% normal goat serum) for 30 minutes. Slides were then washed and treated with NovaRED substrate (Vector Laboratories) according to the manufacturers instructions, lightly counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

Immunofluorescence Staining to Detect Kupffer Cells

Cryosections prepared from snap-frozen liver tissue blocks were fixed for 5 minutes in room temperature acetone and washed with PBS. All subsequent steps were done at room temperature with four PBS washes after each step. Sections were treated with 10% normal goat serum in PBS for 10 minutes and then with antibodies diluted in PBS + 1% normal goat serum as follows: mouse anti-rat ED2 (2.5 μg/mL; Serotec, Raleigh, NC) or mouse IgG control (Zymed, South San Francisco, CA) for 1 hour, followed by TRITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (1:200; Jackson ImmunoResearch) for 30 minutes. Sections were then blocked for 10 minutes with PBS + 10% normal mouse serum and treated with mouse anti-rat ED1-FITC (2.5 μg/mL in PBS + 1% normal mouse serum; Serotec) or mouse IgG-FITC control (Jackson ImmunoResearch) for 30 minutes. After washing, sections were fixed with 10% neutral-buffered formalin and mounted with Vectashield (Vector Laboratories).

Isolation and Culture of Primary Rat Liver Cells

Primary hepatocytes and nonparenchymal cells (NPC) were obtained by perfusing the livers of sodium pentobarbital-anesthetized rats with a solution of 0.05% collagenase IV (Worthington Biochemical Corp., Lakewood, NJ) according to the two-step method of Seglen (17). Dissociation of the liver tissue, preparation of a single-cell suspension and subsequent purification steps were done at 4°C. Viability of the initial suspension ranged from 70% to 85%. Hepatocytes were separated from NPC by centrifugation at 50 × *g* for 2.5 minutes and further purified by centrifugation on 60% Percoll gradients as detailed by Smedsrod and Pertoft (18). The NPC fraction, retained in the supernatant of the initial differential centrifugation step, was further purified by centrifugation on a two-step Percoll gradient consisting of 60% and 25% layers (18). After centrifugation at 400 × *g* for 20 minutes, cells at the interface of the two layers, consisting of viable NPC, were collected. Hepatocytes were washed, resuspended in hepatocyte culture medium (Biowhittaker, Walkersville, MD) and seeded at 2.5×10^4 per well in 96-well dishes coated with type I rat tail collagen (Sigma-Aldrich). After 2 hours at 37°C, 5% CO_2, the wells were gently washed once and fresh medium was added. Cells were rested for 24 hours prior to assays. NPC were washed and seeded in 96-well dishes at 5×10^4 per well in Williams' medium E supplemented with 10% fetal bovine serum, glutamine and penicillin-streptomycin. After overnight incubation, medium was gently aspirated and fresh medium was added in preparation for the cytotoxicity assay. Alternatively, NPC were seeded at 3×10^6 per 35 mm dish and incubated for 3 hours at 37°C, 5% CO_2. Dishes were washed thrice to remove nonadherent cells, and fresh medium was added. After an overnight incubation, the cells were rinsed and treated with 0.05% trypsin in serum-free medium for 30 minutes at 37°C to further enrich for KC, which are resistant to light trypsin treatment (19). After the addition of fresh medium, cells were cultured overnight. The remaining cells had typical KC morphology and phagocytosed 1 μmol/L fluorescent latex beads.

Primary Cell Cytotoxicity Assays

Ninety-six well cytotoxicity assays were done as per the protocol for tumor cells except that after 24 hours, [^{3}H]leucine (L-leucine [3,4,5-^{3}H]; MP Biomed, Inc., Irvine, CA) was added in leucine-free Williams' medium E containing 5% dialyzed fetal bovine serum (Life Technologies, St. Paul, MN), and the cultures were incubated for an additional 18 hours. Plates were subject to one cycle of freezing and thawing to detach adherent cells prior to harvesting onto filter mats. Results are expressed as cpm ± SD of [^{3}H]leucine incorporated by triplicate wells of cells at the indicated doses of fusion toxin, and are representative of at least two experiments for each cell type. For qualitative analysis, experiments were run in parallel with KC isolated and cultured in 35 mm dishes as described above. Cells were incubated with 1 nmol/L fusion toxins, and after 24 hours, monolayers were fixed, stained with 4′,6-diamidino-2-phenylindole and evaluated for cytopathic effects by light and fluorescence microscopy.

Immunofluorescence to Detect Sinusoidal Endothelial Cells

Cryosections were stained for sinusoidal endothelial cells (SEC) using the method described for KC except that the primary antibody was monoclonal anti-rat hepatic sinusoidal endothelial cells, SE-1, 5 μg/mL (KMI Diagnostics/IBL America, Minneapolis, MN; ref. 20), followed by rhodamine-conjugated secondary antibody as above.

Results

In vitro Cytotoxicity of Fusion Toxins

For the *in vivo* studies presented in this report, we utilized four different DT fusion proteins: DT_{388}GMCSF,

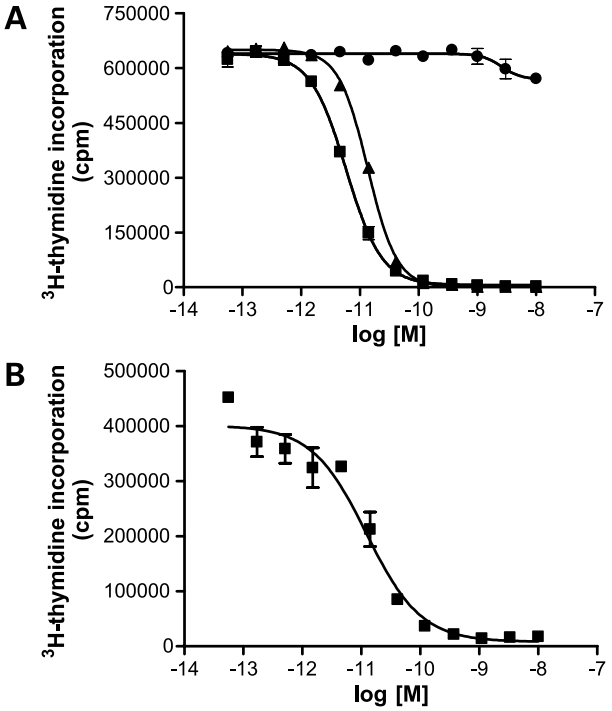

Figure 1. Tumor cells were treated for 72 hours with the indicated doses of fusion toxins and cytotoxicity was assessed by measuring the incorporation of radiolabeled thymidine by viable cells during the last 18 hours of culture. Results are expressed as the cpm±SD of [^{3}H]thymidine incorporated by triplicate wells of cells. **A,** J774: DT_{390}mGMCSF (▲), DTU2mGMCSF (■), and DT_{388}GMCSF (●). **B,** FBL-3: DT_{390}mIL-3 (■).

DT_{390}mGMCSF, DTU2mGMCSF, and DT_{390}mIL-3. As shown in Fig. 1, all fusion proteins were highly cytotoxic *in vitro* for tumor cells expressing their respective target receptors. The EC_{50} for each fusion toxin was as follows: DT_{390}mGMCSF, 13 pmol; DTU2mGMCSF, 6 pmol; DT_{390}mIL-3, 14 pmol. Note that due to minimal cross-reactivity with the rodent GMCSF receptor, DT_{388}GMCSF did not kill the mouse tumor cell line J774A.1. The EC_{50} of DT_{388}GMCSF for TF1vSrc human AML cells is approximately 1.0 pmol (13).

In vitro Toxicity: Serum Chemistry

Rats were treated with 200 μg/kg fusion toxins and hepatic transaminase levels were measured in serum 16 hours later. As shown in Fig. 2, DT_{390}mGMCSF treatment induced a significant increase in serum AST and ALT levels, approximately 15-fold ($P < 0.01$) and 4-fold ($P < 0.01$), respectively, relative to saline-treated controls. In addition, AST and ALT levels were significantly higher in DT_{390}mGMCSF treated rats as compared with DTU2 mGMCSF, DT_{390}mIL-3, and DT_{388}GMCSF-treated rats ($P < 0.01$). Small increases in transaminase levels relative to saline-treated controls were observed for DT_{390}mIL-3, DTU2mGMCSF, and DT_{388}GMCSF, but these increases were not statistically significant. Other serum chemistries were normal in DT_{390}mGMCSF-treated rats with the exception of alkaline phosphatase and lactate dehydrogenase, which were elevated an average of 2- and 25-fold, respectively, relative to controls (data not shown). Peripheral blood cell counts were within reference range in DT_{390}mGMCSF-treated rats (data not shown). Therefore, DT_{390}mGMCSF caused liver injury in rats similar to that which is observed with DT_{388}GMCSF in humans. In addition, these results show that hepatotoxicity is GMCSF receptor–mediated.

In vitro Toxicity: Liver Histology

Elevated serum transaminase levels did not reflect overt pathologic changes in the livers of DT_{390}mGMCSF-treated rats as assessed by H&E staining of formalin-fixed, paraffin-embedded tissue. Figure 3B depicts liver tissue sampled from a DT_{390}mGMCSF-treated rat that had a serum ALT level of 490 IU/L, as compared with that of the saline-treated control in Fig. 3A, which had an ALT level of 45 IU/L. There was no evidence for hepatocyte death or inflammatory infiltrate and liver architecture

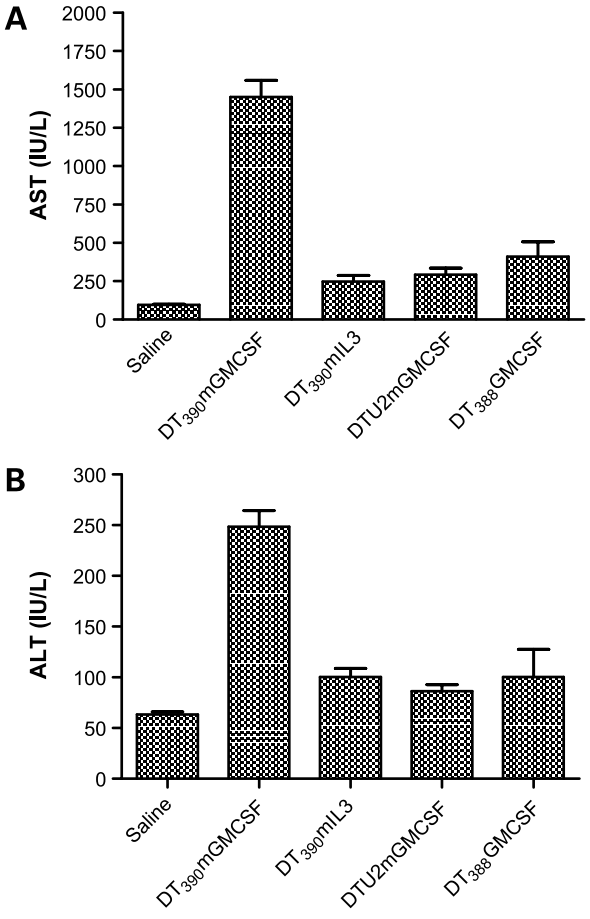

Figure 2. Rats were treated with 200 μg/kg DT_{390}mGMCSF, DT_{388}GMCSF, DT_{390}mIL-3, DTU2mGMCSF, or saline. Serum was collected after 16 hours and analyzed for levels of AST (**A**) and ALT (**B**). Results are expressed as the mean±SD of AST or ALT (international units per liter) in serum. Saline ($n = 12$), DT_{390}mGMCSF ($n = 14$), DT_{390}mIL-3 ($n = 8$), DTU2mGMCSF (n = 8), and DT_{388}GMCSF ($n = 4$).

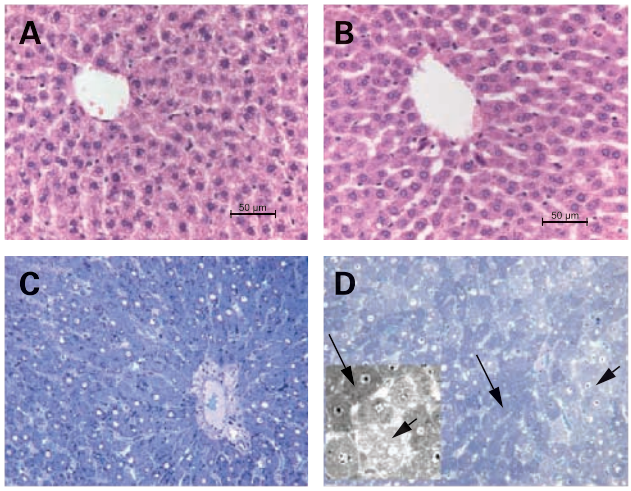

Figure 3. Rats were treated with saline (**A, C**) or with 200 μg/kg DT_{390}mGMCSF (**B, D**). Livers were harvested 12 to 18 hours later for histologic analysis. **A** and **B,** formalin-fixed, paraffin-embedded tissue stained with H&E; magnification (× 170). **C** and **D,** glutaraldehyde-fixed, plastic-embedded tissue stained with toluidine blue. *Short arrow,* lightly stained hepatocytes; *long arrow,* darkly stained hepatocytes; magnification (× 130). *Inset*, high-magnification image (× 315) in grayscale to highlight the heterogeneous staining pattern in DT_{390}mGMCSF-treated liver.

remained largely intact. However, light microscopy analysis of hepatic tissue embedded for electron microscopy revealed a heterogeneous pattern on staining with toluidine blue, suggestive of hepatocyte swelling in DT_{390}mGMCSF-treated livers (Fig. 3D and *inset*). This pattern was not detected in saline-treated control livers (Fig. 3C). Ultrastructural analysis confirmed that swollen hepatocytes were interspersed among hepatocytes with normal morphology in livers from treated but not control rats (Fig. 4A and B). In no case did we find evidence, at the light or ultrastructural level, for hepatocyte apoptosis or necrosis. Therefore, DT_{390}mGMCSF treatment causes hepatocellular swelling, which likely explains hepatic enzyme elevations observed in the serum of treated rats.

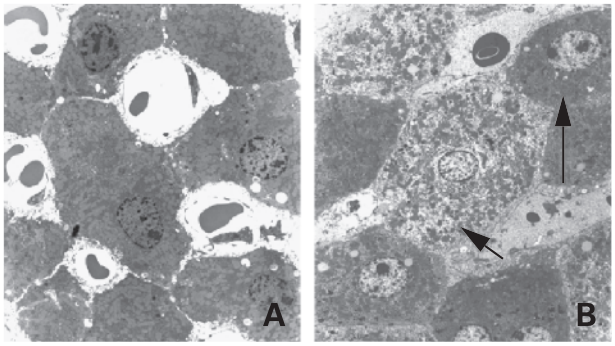

Figure 4. Rats were treated with saline (**A**) or 200 μg/kg DT_{390} mGMCSF (**B**). Livers were harvested, fixed and processed for electron microscopy 18 hours later. *Short arrow,* swollen hepatocyte; *long arrow,* hepatocyte with normal morphology; magnification (× 1,200).

Immunohistochemical Staining for Apoptotic Cells in the Liver

DT_{388}GMCSF has been shown to induce death by apoptosis in susceptible cells (10, 21). In order to evaluate DT_{390} mGMCSF-treated livers more closely for the presence of apoptotic cells, we stained liver sections with an antibody specific for the activated form of caspase-3 (22). As shown in Fig. 5, apoptotic cells (*stained red*) were detected in the sinusoidal spaces of DT_{390}mGMCSF-treated but not saline-treated rats. In contrast, parenchymal cells did not exhibit specific staining with this antibody. These results indicate that sinusoidal cells, not hepatocytes, are direct targets of DT_{390}mGMCSF.

Immunofluorescence Staining for ED1-Positive and ED2-Positive Cells in the Liver

DT_{388}GMCSF was designed to target GMCSF receptor-positive leukemic blasts. We have shown that DT_{390} mGMCSF-induced liver toxicity requires specific interaction with GMCSF receptor-positive cells and that sinusoidal cells in the livers of treated rats die by apoptosis. Therefore, we examined the livers of DT_{390}mGMCSF-treated rats for the presence of KC, resident liver macrophages that are located in the sinusoidal spaces. The monoclonal antibody ED1 recognizes a lysosomal membrane protein expressed by all subsets of rat monocytes and macrophages, whereas ED2 expression is restricted to resident tissue macrophages, including KC (23). As shown in Fig. 6, ED1-positive and ED2-positive cells were virtually depleted in the livers of DT_{390}mGMCSF-treated rats. DT_{390}mGMCSF-treated livers displayed a punctate staining pattern, likely reflecting remnants of dead KC. Intact ED1-positive and ED2-positive cells were rarely observed. In contrast, ED1-positive and ED2-positive cells were detected in the livers of DT_{390}mIL-3 and DTU2mGMCSF-treated rats. Therefore, DT_{390} mGMCSF targets and depletes KC in the rat model. In contrast, DT_{390}mIL-3 and DTU2mGMCSF do not effectively target KC *in vivo*.

Susceptibility of Primary Liver Cells to DT_{390}GMCSF *In vitro*

To confirm that DT_{390}mGMCSF is directly cytotoxic for KC but not hepatocytes, and to establish an *in vitro* system

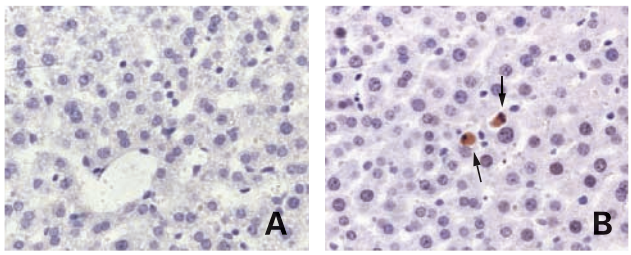

Figure 5. Rats were treated with saline (**A**) or 200 μg/kg DT_{390} mGMCSF (**B**) for 12 hours. Livers were harvested and sections were prepared and stained with rabbit anti-activated caspase-3 followed by goat anti-rabbit IgG-horseradish peroxidase. Caspase-3-positive cells were visualized using the chromogenic horseradish peroxidase substrate, NovaRED. Controls treated with rabbit IgG in place of the first antibody showed no positive staining (data not shown); magnification (× 265).

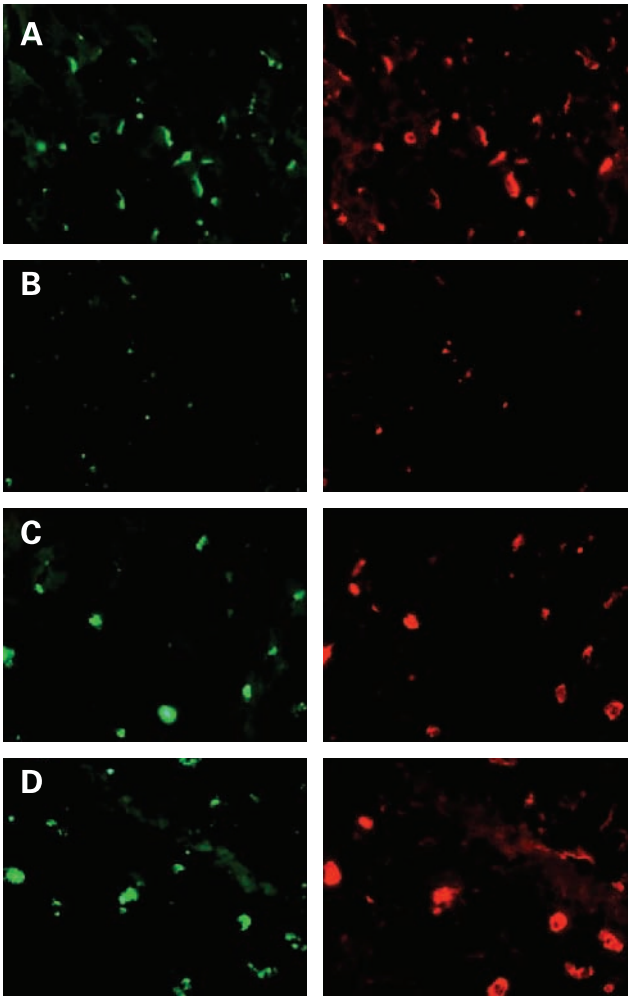

Figure 6. Rats were treated with saline (**A**), DT_{390}mGMCSF (**B**), DT_{390} mIL-3 (**C**), or DTU2mGMCSF (**D**). Livers were harvested 16 hours later and snap-frozen in optimum cutting temperature embedding compound. Cryosections were prepared and stained with monoclonal anti-rat ED2 followed by goat anti-mouse IgG-rhodamine or anti-rat ED1 directly conjugated to FITC. Controls treated with mouse IgG in place of the first antibody showed no specific staining (data not shown); magnification (× 230).

for further study, we isolated primary hepatocytes and KC-enriched NPC from normal rats and cultured the cells in the presence of fusion toxins. Ricin, included as a positive control, killed primary hepatocytes in a dose-dependent manner, whereas DT_{390}mGMCSF had no effect on hepatocytes (Fig. 7A). Similar results were obtained with primary rat hepatocytes obtained from a commercial source (Biowhittaker) and with the rat hepatoma McA-RH7777 (data not shown).

In contrast to the insensitivity of hepatocytes to its cytotoxic effects, DT_{390}mGMCSF inhibited the incorporation of [^{3}H]leucine in the KC-containing NPC fraction in a dose-dependent manner (Fig. 7B). In addition, DTU2mGMCSF was significantly less cytotoxic for the KC-containing NPC fraction than DT_{390}mGMCSF (EC_{50}: 1–3 nmol/L, DTU2GMCSF; 4.4 pmol, DT_{390}GMCSF; Fig. 7B).

KC that were further purified from the NPC fraction by adherence and light trypsin treatment to remove contaminating SEC were killed after 18 hours of culture with 1 nmol/L DT_{390}mGMCSF (Fig. 8C). KC that were treated with the same dose of DT_{388}GMCSF (Fig. 8B) were intact, similar to controls incubated without toxins (Fig. 8A). 4′,6-Diamidino-2-phenylindole–staining revealed nuclei with apoptotic morphology in DT_{390}mGMCSF-treated cells (Fig. 8E, G). These results are consistent with our *in vivo* data demonstrating that DT_{390}GMCSF is directly cytotoxic for KC. Furthermore, our results indicate that the modified fusion toxin, DTU2mGMCSF, is not hepatotoxic because it does not effectively target KC.

Immunofluorescence Staining for Sinusoidal Endothelial Cells

Having established that KC but not hepatocytes are directly killed by DT_{390}mGMCSF, we addressed the possibility that DT_{390}GMCSF targets the third major liver cell population, the SEC. Fresh-frozen liver samples were stained with monoclonal antibody SE-1 which is specific for rat hepatic SEC (20). No difference in staining pattern or intensity was observed between saline-treated controls and DT_{390}mGMCSF-treated livers (Fig. 9). As reported

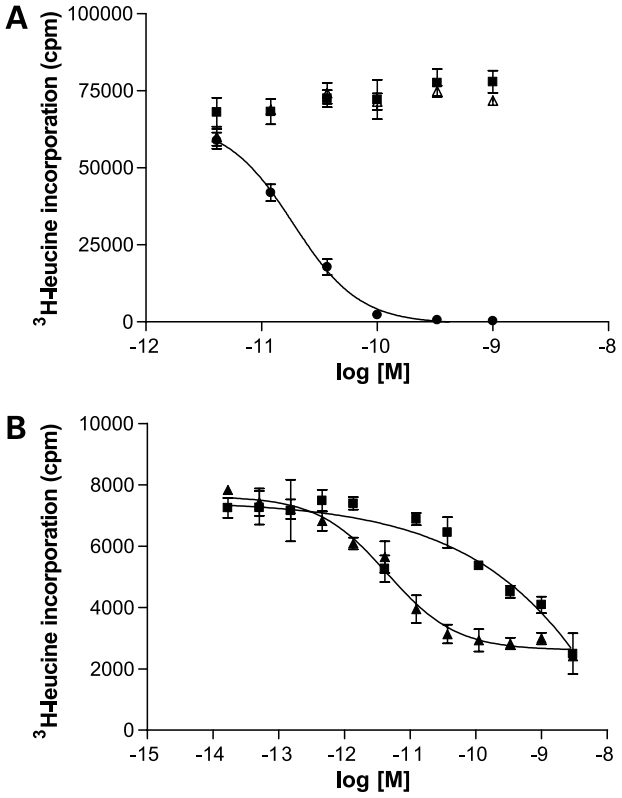

Figure 7. Primary rat hepatocytes (**A**) and NPC (**B**) were isolated as described in Materials and Methods. The day after plating, cells were fed with fresh medium containing fusion toxins as indicated: **A,** DT_{390} mGMCSF (△), DT_{388}GMCSF (■), or ricin (●); **B,** DT_{390}mGMCSF (▲), or DTU2mGMCSF (■). Cytotoxicity was assessed at 24 hours by measuring the incorporation radiolabeled leucine (cpm ± SD of triplicate wells) by viable cells.

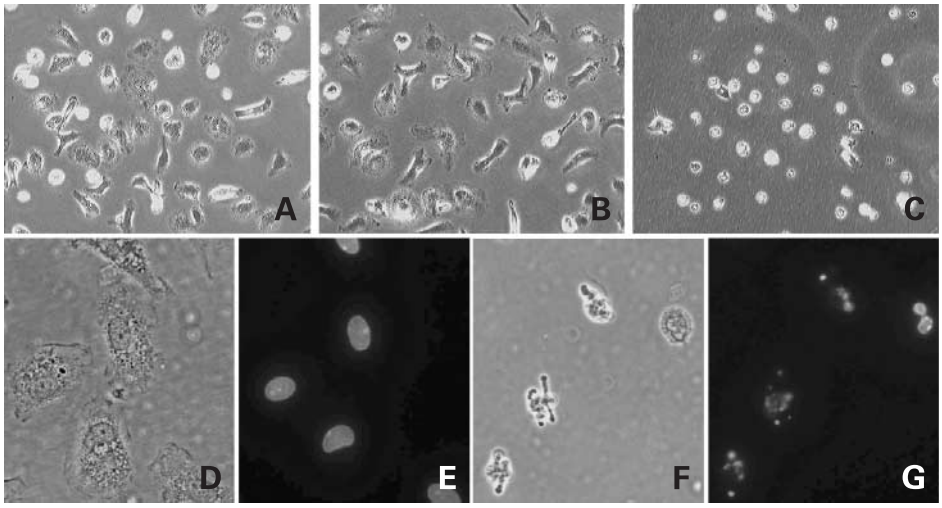

Figure 8. Phase contrast photomicrographs of KCs purified from the NPC fraction by adherence as described in Materials and Methods, and cultured for 18 hours in medium without toxin (**A**), medium containing 1 nmol/L DT_{388}GMCSF (**B**), or medium containing 1 nmol/L DT_{390}mGMCSF (**C**); magnification (× 125). Phase contrast and corresponding 4′,6-diamidino-2-phenylindole–stained images of cells treated with saline (**D, E**) or DT_{390}mGMCSF (**F, G**) as described for **A** and **B**; magnification (× 250).

previously for this antibody, SECs (*short arrow*), but not endothelium lining the large vessels (*long arrow*), stained positive for SE-1 (20). Although this analysis does not rule out functional effects on SEC, it indicates that unlike KC, SEC remain intact in DT_{390}mGMCSF-treated livers.

Discussion

There are currently few therapeutic alternatives for the treatment of relapsed or refractory AML. Ongoing phase I clinical trials with DT_{388}GMCSF have shown antileukemic efficacy, but the use of the drug is limited by its hepatotoxic effects. Defining the mechanism of hepatotoxicity would aid in the rational design of new fusion toxins and could lead to the development of therapies to block the undesirable effects on the liver. To this end, we developed a rat model using the rodent-tropic fusion toxin DT_{390}mGMCSF, and two related DT-conjugated fusion toxins, DT_{390}mIL-3 and DTU2mGMCSF. Similar to the effects of DT_{388}GMCSF in humans, DT_{390}mGMCSF induced significant serum AST and ALT elevations in Sprague-Dawley rats. This model allowed us to study the effect of DT-conjugated fusion toxins on rat liver cell populations *in vivo* and *in vitro*.

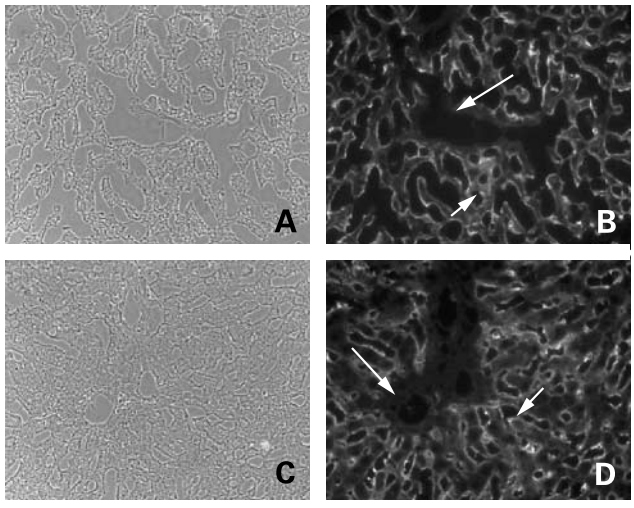

Figure 9. Rats were treated i.p. with saline (**A, B**) or 200 μg/kg DT_{390}mGMCSF (**C, D**). Livers were harvested after 16 hours and snap-frozen in optimum cutting temperature embedding compound. Cryosections were prepared and stained with monoclonal anti-rat hepatic SECs (SE-1) followed by rhodamine-conjugated goat anti-mouse IgG. Phase contrast images (**A, C**) are shown for each fluorescent field (**B, D**) to highlight tissue architecture. *Short arrows,* endothelial cells lining the sinusoids but not the large vessels; *long arrows*, stain with SE-1. Magnification (× 170).

Using the rat model, our initial *in vivo* observation was that despite 4- to 15-fold elevations in ALT and AST, respectively, livers from treated animals were histologically unremarkable as assessed by H&E staining (Fig. 3). There was no evidence for hepatocyte apoptosis/necrosis or inflammation, even when we employed a dosing regimen in which the toxin was given daily over a period of several days (data not shown). This is in marked contrast to other hepatotoxic agents, such as CCL4 and acetaminophen, which induce large-scale hepatocyte necrosis accompanied by up to 100-fold elevations in serum transaminases (24, 25). The relatively moderate transaminase elevations induced by DT_{390}mGMCSF are likely due to the release of cytoplasmic contents from swollen hepatocytes, which were revealed when tissues were fixed and processed for ultrastructural analysis. We hypothesize that in the rat model, hepatocellular swelling does not progress to overt liver damage because of the well-documented ability of hepatocytes to recover from this type of injury. For example, hepatocellular swelling can be induced experimentally in rats by the presence of excess glutamine, an effect which is fully reversible (26). Indeed, in most cases, transaminemia observed in human patients treated with DT_{388}GMCSF is transient (7) suggesting that hepatocytes recover. In some cases, DT_{388}GMCSF-mediated hepatotoxic effects may be amplified by factors related to AML and prior chemotherapy, leading to liver failure.

Consistent with the lack of *in vivo* evidence for hepatocyte apoptosis/necrosis, we have shown that primary rat

hepatocytes (Fig. 7) and a rat hepatocyte cell line (data not shown) are not killed by DT_{390}mGMCSF *in vitro*. These results corroborate our published data demonstrating that DT_{388}GMCSF does not kill the human hepatocyte cell line, HepG2 (7). Therefore DT_{390}mGMCSF seems to induce liver injury via an indirect rather than a direct effect on hepatocytes.

In contrast to the insensitivity of hepatocytes to DT_{390}mGMCSF, we have provided multiple lines of evidence that DT_{390}mGMCSF targets GMCSF receptor–positive liver macrophages (KC). ED1-positive and ED2-positive KC were depleted in the livers of treated rats, and apoptotic cells were detected in the sinusoids. In addition, primary KC isolated from normal rats were killed by DT_{390}mGMCSF *in vitro*. Unlike KC, our data indicate that SEC do not seem to be directly targeted by DT_{390}mGMCSF *in vivo*.

We have shown that KC are targeted by DT_{390}mGMCSF and further, that hepatotoxicity is a direct result of the targeting of GMCSF receptor-positive cells, rather than a nonspecific effect mediated by the DT portion of the molecule. Treatment of rats with DT_{390}mIL-3, which targets the rodent IL-3 receptor, or DT_{388}GMCSF, which does not cross-react with the rat GMCSF receptor, did not induce transaminase elevations of a magnitude comparable that of DT_{390}mGMCSF. A direct relationship between DT_{390}mGMCSF-induced KC death and liver injury was further substantiated by treating rats with the modified fusion toxin, DTU2mGMCSF. In marked contrast to DT_{390}mGMCSF, treatment of rats with DTU2mGMCSF did not damage the liver as reflected by serum transaminases, and did not deplete KC *in vivo*. Furthermore, DTU2mGMCSF was significantly less effective at killing primary KC *in vitro* than was DT_{390}mGMCSF. Although not formally addressed in this study, these results suggest that KC have insufficient levels of cell surface urokinase activity to confer susceptibility to DTU2mGMCSF. A more detailed analysis of the interaction of DTU2mGMCSF with KC will be a subject of future studies.

The current report does not address the molecular mechanism by which KC death leads to hepatocyte injury. However, our analysis suggests that hepatocytes in treated animals suffer membrane damage that alters the ionic balance and causes swelling, which likely accounts for the release of hepatic transaminases into serum. This effect could be mediated by the local release of hepatotoxic factors from dying KC. Indeed, we have evidence for the presence of nitrotyrosine-modified proteins in the livers of treated rats, suggesting that superoxide and nitric oxide might be involved in the toxicity mechanism.[6] Alternatively, as has been reported for acetaminophen hepatotoxicity (27), the loss of KC may deplete a hepatoprotective cytokine such as IL-10 or IL-6, or a cyclooxygenase-derived prostanoid. These two possibilities are not mutually exclusive. Current studies are focused on *in vivo* and *in vitro* approaches using the rat model to address these issues.

[6] M.M. Westcott and A.E. Frankel, unpublished data.

Others have reported liver toxicity associated with high doses of DTmGMCSF in leukemic Brown Norway rats but the mechanism of liver injury was not addressed in the study (28). We have reported a Sprague-Dawley model of DT_{388}GMCSF-induced liver damage that has revealed a critical role for KC in this process, and which should allow us to determine how the targeting of KC leads to hepatocyte injury. Once this mechanism is understood, the Brown Norway model can be used to address the effect of leukemia on drug-induced hepatotoxicity. The importance of understanding the relationship between KC injury and liver damage extends to other immunotoxins, for example, anti-CD33 calichaemicin (Mylotarg). This drug, also used in the treatment of AML, induces liver damage by an unknown mechanism that may be related to its targeting of CD33-positive liver macrophages (29).

In conclusion, the data presented in the current report have significantly advanced our understanding of DT_{388}GMCSF-mediated liver damage. In addition, we have described two fusion toxins that do not have significant hepatotoxic effects in rats, and may therefore prove to be safer alternatives for the treatment of AML. Indeed, DT_{388}IL-3, the human equivalent of DT_{390}mIL-3 showed little to no toxicity in preclinical dose-escalation studies (30) and its antileukemic efficacy is currently being evaluated in phase I clinical trials. DTU2mGMCSF, unlike DT_{390}mGMCSF, did not induce liver injury in rats due to its inefficient targeting of KC. Thus, a single substitution designed to enhance tumor cell specificity virtually eliminated hepatotoxicity *in vivo*. These results, along with our recent report describing the *in vitro* antitumor activity of DTU2GMCSF (13) provide initial validation for the dual specificity approach to the design of fusion toxins. Current studies are focused on confirming *in vivo* safety and evaluating the antileukemic efficacy of DTU2mGMCSF in a murine leukemia model. Further characterization of DTU2mGMCSF, combined with a more detailed analysis of the mechanism of DT_{390}mGMCSF-induced liver toxicity, should lead to significant advances in the treatment of relapsed and refractory AML.

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