DIPHTHERIA TOXIN FUSED TO VARIANT HUMAN INTERLEUKIN-3 INDUCES CYTOTOXICITY OF BLASTS FROM PATIENTS WITH ACUTE MYELOID LEUKEMIA ACCORDING TO THE LEVEL OF INTERLEUKIN-3 RECEPTOR EXPRESSION

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Abstract
Leukemic blasts from patients with acute myeloid leukemia (AML) frequently express high levels of the interleukin-3 receptor α chain (IL-3Rα). In the present study, we have explored the sensitivity of primary leukemic blasts obtained from 34 AML patients to a diphtheria toxin (DT) composed of the catalytic and translocation domains of DT (DT388) fused to IL-3 (DT388IL-3) and to DT388 fused to a variant IL-3 with increased binding affinity (DT388IL-3[K116W]). On a molar basis, DT388IL-3[K116W] was significantly more active than DT388IL-3 in mediating leukemic cell killing. The rate of cell killing induced by the two DT/IL-3 fusion proteins was significantly correlated with the level of IL-3Rα/IL-3Rβ expressed on leukemic blasts. These observations support a potential use of DT388IL-3[K116W] in the treatment of refractory AMLs and provide a simple biochemical parameter for the selection of eligible patients.
INTRODUCTION

Acute myeloid leukemia (AML) encompasses a group of heterogeneous disorders characterized by the expansion of malignant clones of hematopoietic cells blocked at various stages of differentiation. Several lines of evidence suggest that multiple molecular mechanisms are involved in the pathogenesis of AMLs (1). For example, chromosome translocations resulting in fusion genes that generate new proteins have been shown to play a major role in the genesis of leukemias (2). However, for development of overt leukemia condition, additional mutations and phenotypic abnormalities are required. Among them, a key role is played by mutations or alterations in the level of expression of membrane receptors involved in the control of the proliferation of hemopoietic cells, such as flt3, c-kit and IL-3R.

Flt3 is mutated and/or overexpressed in 30-40% of AMLs (3), while the IL-3R is overexpressed in about 45% of AMLs (4). Furthermore, the analysis of an AML subpopulation enriched in leukemic stem cells showed that these cells express elevated levels of IL-3Rα, while in the normal counterpart low levels of IL-3Rα have been observed, thus indicating that the IL-3Rα is a unique marker of leukemic stem cells (5). According to these findings it has been suggested that the IL-3R may represent an important target for the development of new antileukemic drugs (6).

A genetically engineered fusion toxin composed of the first 388 amino acid residues of diphtheria toxin (DT) with an H-M linker fused to human IL-3 (7). This IL-3 DT was found to be toxic towards leukemic blasts (8) and in vivo studies in Cynomolgus monkeys have shown that it is relatively well tolerated up to 100 μg/Kg (9, 10). These results contrast with the limited tolerance to the GMCSF DT fusion protein (DT388GMCSF) which produced liver injury above 4 - 7 μg/kg in monkeys and AML patients (11, 12). Preclinical studies with rodent cell directed DT fusion proteins
(DT<sub>388</sub>mGMCSF and DT<sub>388</sub>mIL-3) showed Kupffer cell-mediated liver injury for the GMCSF DT fusion protein but not the IL-3 DT fusion protein consistent with the presence of GMCSFR but not IL3R on the target cells (13). Based on these findings, a phase I clinical trial has recently been initiated with DT<sub>388</sub>IL-3 for the treatment of AML patients. To date, six AML patients have been treated with DT<sub>388</sub>IL-3 without evidence of severe toxicities or liver damage (14).

Finally, with the aim of improving antileukemic activity, diphtheria toxin has been fused to IL-3 variants. Based on mutagenesis studies by several laboratories, substitution of a large, bulky hydrophobic residue (tryptophan) at IL-3 position 116 enhanced the hydrophobic interaction with IL-3R<sub>α</sub>. We prepared the modified DT<sub>388</sub>IL-3[K116W] and the resulting fusion protein exhibited an enhanced binding to the IL-3R and more potent cytotoxicity towards leukemic cell lines (15).

Using primary blasts from AML patients in vitro, we compared in the present study the anti-leukemic cytotoxic activity of the DT<sub>388</sub>IL-3 and that of the DT<sub>388</sub>IL-3[K116W]. We found that the variant IL-3 fusion protein exhibited a more potent anti-leukemic activity than the WT IL-3 fusion protein.

**MATERIALS AND METHODS**

**Cells**

Fresh leukemic blasts from 34 AML patients, obtained after informed consent, were isolated from either bone marrow or peripheral blood by Ficoll-Hypaque density gradient centrifugation and were immediately processed. All patients were consecutively diagnosed at the Division of Hematology, Department of Cellular Biotechnologies and Hematology of the University “La Sapienza”, Rome, Italy. Leukemias were classified by
morphologic criteria according to the FAB classification and samples contained more than 70% leukemic blasts.

Approval for these studies was obtained from the institutional review board. Informed consent was obtained according to the Declaration of Helsinki.

**Preparation of DT$_{388}$IL-3**

The preparation and purification of DT$_{388}$IL-3 and DT$_{388}$IL-3[K116W] has been previously reported in detail (7, 8).

**Hematopoietic growth factor receptor expression**

Phycoerythrin (PE)–labelled anti-IL-3R$\alpha$ chain (CD123) monoclonal antibody clones 7G3 and 9G5 were purchased from Pharmingen (San Diego, CA); PE-labelled anti-IL-3R$\beta$ chain (CDw131) monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Negative controls were represented by isotype-matched PE-labelled mouse immunoglobulins. Cells were incubated with optimal concentrations of the antibodies and, after two washings in cold PBS, were analyzed for fluorescence by flow cytometry (FACSCAN, Becton Dickinson, San Diego CA) by Cell Quest Software.

**In vitro incubation of leukemic blasts with IL3 diphtheria fusion proteins and study of apoptosis**

Leukemic cells were grown in vitro in freshly prepared Iscove’s-modified minimal essential medium (IMDM, Gibco, New York, NY) containing 10% fetal calf serum (Gibco) at a final concentration of 5x10$^5$ cells/ml either in the absence (control) or in the presence of either DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W] at various concentrations (from 10$^{-11}$M to 10$^{-7}$M). At various days of culture, one cell aliquot was removed and assayed by FACS analysis for the presence of apoptotic cells using the Annexin V binding assay.

**RESULTS AND DISCUSSION**

Newly diagnosed AML patients were investigated for their in vitro sensitivity to the DT fused to the wild-type IL-3 (DT$_{388}$IL-3) and to the DT fused to
variant IL-3 (DT$_{388}$IL-3[K116W]). Leukemic blasts from 2 M0, 4 M1, 8 M2, 2 M3, 10 M4 and 8 M5 were incubated in vitro for 24-48 hours either in the absence (Control) or in the presence of increasing concentrations (from 1x10$^{-11}$ to 1x10$^{-7}$ M) of either DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W] and the percentage of apoptotic cells was measured by the AnnexinV/PI labelling. A representative experiment reported in Fig. 1 A clearly indicated that maximal cytotoxicity was observed using the 1x10$^{-7}$M concentration of the two toxins and that DT$_{388}$IL-3[K116W] was more active than DT$_{388}$IL-3 in killing AML blasts.

We therefore tested the effects of the two fusion proteins at 1x10$^{-7}$M on AML cells. The results of this analysis showed that after 24 hours of incubation the DT$_{388}$IL-3[K116W] was more active than the DT$_{388}$IL-3 in inducing apoptosis of leukemic blasts (52.4±4.5 vs 27.1±3.5, p<0.0001). (Fig. 1 B).

It is of interest to note that the rate of cell killing induced by these diphtheria IL-3 fusion proteins varied among the different AML cases tested. In order to understand the origin of this heterogeneity we investigated whether the rate of blast cell killing induced by the IL-3 diphtheria fusion proteins was dependent upon the levels of IL-3R expression on leukemic cells. For this purpose, the expression of IL-3R$\alpha$ (CD123) and IL-3R$\beta$ (CDw131) was analyzed by flow cytometry using specific monoclonal antibodies conjugated with phycoerythrin. This analysis showed that the levels of IL-3R$\alpha$ and IL-3R$\beta$ expressed on AML cells are heterogeneous and that they clearly correlated with the percentage of apoptotic cells induced by the two IL-3 diphtheria fusion proteins (Fig. 1 C and D).

AML cases expressing very low or low levels of IL-3R$\alpha$/IL-3R$\beta$ showed only a very low/low rate of cell killing after 24/48h of incubation with the two IL-3 diphtheria fusion proteins (data not shown); AML cases expressing moderate IL-3R$\alpha$/IL-3R$\beta$ levels (see 1 representative example in Fig. 2, top
panels) exhibited a moderate rate of cell killing after 24 hours of incubation with DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W]; AML cases exhibiting high IL-3R$\alpha$ and IL-3R$\beta$ levels (see one representative case in Fig.2, bottom panels) showed a high rate of cell killing after incubation with IL-3 diphtheria fusion proteins, particularly with DT$_{388}$IL-3[K116W].

Based on these observations we conclude that the levels of IL-3R$\alpha$/IL-3R$\beta$ on the surface of AML blasts represent a major determinant in their sensitivity to IL-3 diphtheria toxins.

The results of the present study clearly indicate that the DT$_{388}$IL-3[K116W] exerts a potent in vitro anti-leukemic activity. The cytotoxic activity of this IL-3/diphtheria toxin is particularly pronounced in AML samples expressing high IL-3R$\alpha$/IL-3R$\beta$ levels; such patients are known to have a poor prognosis and a high rate of relapse (4). These patients could thus represent the natural candidates for an anti-leukemic therapy based on the use of DT$_{388}$IL-3[K116].

In summary, our data suggest that the variant DT$_{388}$IL-3[K116W] is a promising new agent for patients with chemotherapy-refractory AML. Because of the persistently poor prognosis of a large proportion of AML patients, the further pre-clinical development of this agent appears warranted.
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FIGURE LEGENDS

**Fig. 1** A: Effect of increasing concentrations of DT$_{388}$IL-3 and DT$_{388}$IL-3[K116W] on the induction of apoptosis in primary AML leukemic blasts. Leukemic cells from 1 representative case have been grown in vitro for 24 hours in the presence of either DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W] and then the percentage of apoptotic cells was determined by the Annexin V binding assay. B: Percentage of apoptotic cells observed on the leukemic blasts of 25 AML patients grown for 24 hours in the presence of either DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W]. C, D: Correlation between the percentage of apoptotic cells induced by 24 hours incubation in the presence of either DT$_{388}$IL-3 or DT$_{388}$IL-3[K116W] and the level of IL-3R$\alpha$ (C) or IL-3R$\beta$ (D) on AML cells, expressed as mean fluorescence intensity (MFI).

**Fig. 2** Flow cytometry analysis of IL-3R$\alpha$ and IL-3R$\beta$ expression (left) and of annexin V/propidium iodide (PI) staining (right) in two representative AML patients.
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Diphtheria toxin fused to variant human interleukin-3 induces cytotoxicity of blasts from patients with acute myeloid leukemia according to the level of interleukin-3 receptor expression

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