Recombinant CD64-Specific Single Chain Immunotoxin Exhibits Specific Cytotoxicity against Acute Myeloid Leukemia Cells

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ABSTRACT

CD64, the high affinity receptor for IgG (FcγRI) is expressed on acute myeloid leukemia blast cells and has recently been described as a specific target for immunotherapy. To generate a recombinant immunotoxin, the anti-CD64 single chain fragment (scFv) m22 was cloned into the bacterial expression vector pBM1.1 and fused to a deletion mutant of Pseudomonas aeruginosa exotoxin A (ETA’). Genetically modified Escherichia coli BL21 Star (DE3) were grown under osmotic stress conditions in the presence of compatible solutes. After isopropyl β-D-thiogalactoside induction, the 70-kDa Hisα-tagged m22(scFv)-ETA’ was directed into the periplasmic space and purified by a combination of metal-ion affinity and molecular-size chromatography. The characteristics of the recombinant protein were assessed by ELISA, flow cytometry, and toxicity assays, using CD64-positive AML cells. Binding specificity of m22(scFv)-ETA’ was verified by competition with the parental anti-CD64 monoclonal antibody m22. The recombinant immunotoxin showed significant toxicity toward the CD64-positive cell lines HL-60 and U937 reaching 50% inhibition of cell proliferation at a concentration (IC50) of 11.6 ng/ml against HL-60 cells and 12.9 ng/ml against U937 cells. Approximately 41% of primary leukemia cells from a patient with CD64-positive AML were driven into early apoptosis by m22(scFv)-ETA’ as measured by flow cytometric analysis. This is the first article documenting the specific cytotoxicity of a novel recombinant immunotoxin with major implications for immunotherapy of CD64-positive diseases.

INTRODUCTION

AML is the most common acute leukemia in adults with an incidence of ~10,000 people/year in the United States (1). AML is characterized by the proliferation of clonal precursor myeloid cells with arrested differentiation (2). The molecular and biological evolution of these malignant clones occurs in a stepwise series of events involving proto-oncogenes, tumor suppressor genes, and interactions with hematopoietic growth factors (3). According to the French-American-British classification system, AML of type M4 and M5 morphology is significantly correlated with expression of the high-affinity receptor for IgG, FcγRI (CD64; Ref. 4). CD64 is a 72-kDa cell surface glycoprotein, which is normally expressed on monocytes/macrophages and dendritic cells (5). The biological functions mediated by this receptor include superoxide and cytokine production (tumor necrosis factor α, IL-1, and IL-6), cytotoxicity, endocytosis/phagocytosis, and support of antigen presentation (6, 7). This receptor represents an appropriate target for immunotherapy of hematological malignancies because it is not present on pluripotent stem and CD34+ hematopoietic progenitor cells, thus guaranteeing regeneration of normal CD64-positive immune effector cells (8).

The ultimate goal in the treatment of cancer patients is the elimination of every tumor cell. Patients with AML have a total of 1012 to 1015 malignant cells at the time of diagnosis (9). Per definition, complete remission is achieved after therapy as soon as <5% of malignant cells are detectable in the bone marrow (10). However, these patients still may carry as much as 1010 malignant cells in the blood stream at this moment. These clinically unidentifiable minimal residual cells are the most common cause of relapse (11). Despite advances in polychemotherapy and radiotherapy, only ~20–30% of patients with AML achieve long-term disease-free survival after first-line therapy (12). Thus, the elimination of minimal residual disease might improve the outcome of patients with AML. Selective approaches, including antibody-based therapies, targeting cytotoxic agents to these cells might offer a promising tool for specific elimination of minimal residual disease (13). To improve the antitumor activity of native antibodies, drugs, isotopes and toxins have been conjugated to mAbs (13).

Recently, a chemically linked anti-CD64 immunotoxin showed rapid binding to and efficient internalization into CD64-positive leukemia cells in vitro and in vivo (14). The authors documented rapid tumor regression of tumor masses ranging from 85 to >90% in a human AML model in NOD/SCID mice. The major obstacle observed in this and other trials were unspecific toxicities, mainly related to the vascular leak syndrome induced by Ricin-A-based chemically linked toxins because of their unspecific binding to endothelial cells (15–17). In recent studies, our group developed a set of recombinant immunotoxins for treatment of Hodgkin’s lymphoma and neuroblastoma consisting of different anti-CD25, anti-CD30 and anti-GD2 scFv antibody fragments genetically linked to Pseudomonas aeruginosa exotoxin A’ (18–20). Having established a very efficient expression protocol (21), the recombinant immunotoxins directly isolated from the periplasmic space of Escherichia coli demonstrated specific antitumor activities in vitro and in vivo. On the basis of this expertise we present here the construction, expression and characterization of the anti-CD64 immunotoxin m22(scFv)-ETA’. Furthermore, we demonstrate the specific activity of this novel immunotoxin against human AML cells.

MATERIALS AND METHODS

Bacterial Strains, Oligonucleotides, and Plasmids. E. coli XL1-blue [supE44 hsdR17 recA1 endA1 gyrA46 thi-1 relA1 lacF’ pro AB’ lacY1 lacZM15 Tn10(Δter)/] was used for propagation of plasmids and E. coli BL21 Star (DE3) [F ompT hsdS2(F’ rK- mK-)] gal dcm recA131 DE3] as host for synthesis of recombinant immunotoxins. Synthetic oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The bacterial expression vector pBM1.1 is derived from the pET27b plasmid (Novagen, Madison, WI) and is used for NH2-terminal fusion of SfiI/NotI-binding structures to the modified deletion mutant of Pseudomonas aeruginosa Exotoxin A (22). Plasmids were prepared by the alkaline lysis method and purified using plasmid preparation kits from Qiagen (Hilden, Germany). Restriction fragments or PCR products were separated by agarose gel electrophoresis and extracted with QIAquick (Qui-
gen). All standard cloning procedures were carried out as described by Sambrook et al. (21).

Patient Samples and Cell Lines. Heparinized peripheral blood samples from an adult patient with AML were obtained after informed consent and with the approval of the clinical research ethics board of the University of Aachen. MNCs were isolated by low-density (<1.007 g/ml) gradient centrifugation using Ficoll-Paque PLUS (Amersham Biosciences, Freiburg, Germany) separation medium. All cell lines, including the CD64-positive AML-derived cell lines HL-60 (provided by Theo. Thepen, Utrecht, the Netherlands) and U937 (DSMZ; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CD64-negative L540Cy (24) and IIA1.6 (provided by DSMZ; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CD64-negative L540Cy (24) and IIA1.6 (provided by Theo. Thepen, Utrecht, the Netherlands) and U937 (DSMZ; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CD64-negative L540Cy (24) and IIA1.6 (provided by Theo. Thepen), were cultured at 37 °C for 1 h. Thereafter, plates were washed, and binding of the °

Construction and Expression of Recombinant m22(scFv)-ETA. The m22(scFv) DNA was amplified from m22-bearing plasmid (provided by T. Thepen) by PCR using the oligonucleotide primers m22(scFv)Back [5'-ATG-GCT-CAG-GGT-GCC-GCC-CAG-CGG-GTT-TTG-GTG-GG-3'] and m22(scFv)For [5'-GAG-TCA-TTC-TGT-TAG-GGC-GCC-CTG-CTG-CC-3']; bold letters: SfiI consensus site, in italics: 5'-m22(scFv) region] and m22(scFv)For [5'-GAG-TCA-TTC-TGT-TAG-GGC-GCC-CTG-GGT-GC-3']; bold letters: NotI consensus site, in italics: 3'-m22(scFv) region]. After SfiI/NotI-digestion, the 754-bp PCR-fragment was cloned into the bacterial expression vector pBM11.1 (22), digested with the same restriction enzymes. The resulting recombinant construct was verified by DNA sequence analysis.

After transformation into BL21 Star (DE3), m22(scFv)-ETA was periplasmically expressed under osmotic stress in the presence of compatible solutes as described by Barth et al. (21). Briefly, recombinant bacteria were harvested 15 h after IPTG induction. The bacterial pellet was resuspended in sonication-buffer [75 mM Tris/HCl (pH 8), 300 mM NaCl, 1 capsule of protease inhibitors/50 ml (Complete, Roche Diagnostics, Mannheim, Germany), 5 mM EDTA, 10% (v/v) glyc erol] at 4 °C and sonicated 6 times for 30 s at 200 °C. m22(scFv)-ETA was purified by IMAC using nickel-nitriolaetic chelating Sepharose (Qiagen) and SEC with Bio-Prep SE-100/17 (Bio-Rad, München, Germany) columns according to the manufacturer’s instructions. Recombinant immuno toxin was eluted with PBS (pH 7.4) and 1 mM NaCl, analyzed by SDS/PAGE, quantified by densitometry (GS-700 Imaging Den sitometer; Bio-Rad) after Coomassie staining in comparison with BSA standards and verified by Bradford assays (Bio-Rad).

SDS-PAGE and Western Blot Analysis. SDS-PAGE and Western blotting were performed as described previously (18). m22(scFv)-ETA was detected by anti-ETA mAb TC-1 (Ref. 25; kindly provided by Darrell R. Galloway, Columbus, OH). Bound antibody was visualized with an alkaline-}

RESULTS

Construction and Expression of m22(scFv)-ETA. PCR-amplified m22(scFv) DNA (Fig. 1A) was directionally cloned into the kanamycin-resistant pBM1.1 expression vector containing an IPTG-
inhibitable lac operator, a pelB signal peptide followed by an enterokinase-cleavable His\_10 tag, and modified ETA (Fig. 1B). The deleted domain 1a of *Pseudomonas* Exotoxin responsible for nonspecific cell binding was thus replaced by CD64-specific m22(scFv). Successful cloning was verified by DNA sequence analysis.

After transformation, recombinant *E. coli* BL21 Star (DE3) clones were cultivated under osmotic stress conditions in the presence of compatible solutes. Recombinant immunotoxin was directed into the periplasmic space and the functional m22(scFv)-ETA\(^\prime\) (\(70\) kDa) directly purified by combination of IMAC and SEC to \(>90\%\) purity. At least \(1\) mg of purified m22(scFv)-ETA\(^\prime\) protein was routinely prepared from \(1\) liter of bacterial shaking cultures (Fig. 2A). Intact recombinant immunotoxin was secreted to the periplasmic compartiment, as visualized by immunoblot using TC-1, an ETA\(^\prime\)-specific mAb (Fig. 2B).

**Binding Properties of m22(scFv)-ETA\(^\prime\).** Coupling of the m22(scFv) coding regions to the truncated ETA\(^\prime\) coding sequences did not affect the binding activity of the V\(_H\)/V\(_L\) antibody format. Purified recombinant anti-CD64 immunotoxin always bound to AML cell membrane fractions but not to CD64-negative L540Cy membranes and corresponding intact cells as measured by CM-ELISA (Fig. 3A) and flow cytometry (Fig. 3B), respectively. CD64 specificity was documented by competitive CM-ELISA experiments: binding of m22(scFv)-ETA\(^\prime\) against CD64-positive HL-60 membrane fractions was inhibited by \(\sim 70\%\) upon addition of \(10\) \(\mu g/ml\) mAb m22 (Fig. 3C).

**In Vitro Cytotoxic Activity.** To characterize the cytotoxic activity of the recombinant anti-CD64 immunotoxin in vitro, we evaluated the proliferation of different target cells after incubation with different amounts of m22(scFv)-ETA\(^\prime\). Growth inhibition of AML-derived cell lines HL-60 and U937 were documented by a XTT-based colorimetric assay. Toxic effects were observed against CD64-positive cells with a calculated median IC\(_{50}\) of \(11.6\) ng/ml on HL-60 cells (Fig. 4A) and \(12.9\) ng/ml on U937 cells, respectively (Fig. 4B). The CD64-negative Hodgkin-derived cell line L540Cy and the murine T-cell leukemia-derived cell line IIA1.6 were not affected by recombinant immunotoxin concentrations of up to \(10\) \(\mu g/ml\).

**Analysis of Apoptosis on Primary AML Cells.** The effects of m22(scFv)-ETA\(^\prime\) on the induction of apoptosis in a freshly prepared population of acute myeloma cells from a patient were examined by flow cytometry. Immunophenotyping revealed \(\sim 90\%\) leukemic cells. The expression of CD64 on the primary cells was verified directly with eGFP-tagged m22(scFv) and in a sandwich approach with m22(scFv)-ETA\(^\prime\) (Fig. 5A). Two color flow cytometric analysis using Annexin-FITC and PI (Fig. 5B) discriminated four populations, viable (Fig. 5B, bottom left quadrant), early apoptotic (Fig. 5B, bottom right quadrant), late apoptotic/necrotic (Fig. 5B, top right quadrant), and necrotic cells (Fig. 5B, top left quadrant). Primary patient-derived CD64-negative leukemic cells treated with m22(scFv)-ETA\(^\prime\) for \(18\) h remained mostly viable (\(\sim 90\%\)). Primary patient-derived CD64-positive AML cells treated with the recombinant immunotoxin after incubation for \(18\) h showed viable (\(\sim 44\%\)), early apoptotic (\(\sim 41\%\)), and late apoptotic/necrotic cell populations (\(\sim 15\%\)).

**DISCUSSION**

In this study, we report the construction of the first recombinant anti-CD64 immunotoxin targeting CD64-positive AML cells (29). The overall expression of CD64 on AML cells is \(\sim 58\%\) (14). To realize the construction of the immunotoxin, we fused the anti-CD64 scFv m22 to a truncated *Pseudomonas* exotoxin A (ETA\(^\prime\)). The major findings to emerge from our study are: (a) functional m22(scFv)-ETA\(^\prime\) was directly isolated from the periplasmic space of *E. coli* cultured under osmotic stress conditions in the presence of compatible solutes and additionally purified by a combination of immobilized metal affinity and molecular size chromatography; (b) m22(scFv)-ETA\(^\prime\) bound to CD64-positive cells as documented by CM-ELISA and flow cytometry; (c) CD64-specific binding activity was shown by competition CM-ELISA using increasing concentrations of parental monoclonal antibody m22; and (d) the recombinant immunotoxin exhibited specific cytototoxic activity toward CD64 receptor-expressing AML-derived cell lines HL-60 and U937 and destroyed CD64-positive patient-derived primary AML cells.

Targeting malignant cells selectively via cell-surface receptors is inherently different from surgery, radiation, and chemotherapy and is often considered a new modality for cancer therapy. Recently, AML...
cells were targeted using both anti-CD33 and anti-GM-CSF immunotoxins. The Food and Drugs Administration recently approved the anti-CD33 immunotoxin Gemtuzumab ozogamicin (Mylotarg) for the treatment of relapsed AML in the United States (30). However, hepatotoxicity, including severe hepatic veno-occlusive disease, has been reported in association with the use of Mylotarg, which may result from targeted delivery of the toxin moiety calicheamicin to CD33-expressing cells found in hepatic sinusoids (31). The fusion toxin DT₃₈₈-GM-CSF combining DT with GM-CSF was evaluated in a Phase I dose-escalation trial in patients with relapsed AML (32). DT₃₈₈-GM-CSF induced complete and partial remissions in chemotherapy-resistant AML-patients but produced liver injury characterized by transient transaminasemia and severe liver dysfunction, which was speculated to be a result of cytokine release from GM-CSF receptor-positive liver Kupffer cells. Thus, additional immunotoxins for AML-targeting alternative cell surface receptors and using different cytotoxic components might be beneficial for patients. Furthermore, very recently no liver damage was observed in carcinoma xenograft-bearing mice after repeated application of the recombinant Pseudomonas exotoxin A-based immunotoxin 4D5MOCB-ETA targeting the epithelial cell adhesion molecule in a dose range up to 500 μg · kg⁻¹ (33).

Additional problems identified in clinical trials with chemically coupled immunotoxins are (a) the development of neutralizing antibodies against both the murine IgG and the toxic moiety resulting in a limited number of application in ~40–60% of the patients (15, 16), and (b) the unspecific cytotoxicity related to unspecific binding of Ricin-A-based toxins to endothelial cells because of their (x)D(y)-motif (17). These problems might, at least in part, be circumvented by using recombinant DNA technology to construct smaller and less immunogenic immunotoxins with reduced unspecific toxicities. Recently, it had been reported in first clinical trials that recombinant scFv- or IL-immunotoxin carrying truncated ETA variants show reduced antibody responses in patients (34, 35).

The most important prerequisite for effective immunotoxin therapy is internalization of target antigen after binding of the immunotoxin to CD33-expressing cells allowing its translocation into the cytosol and cell killing. This internalization behavior was recently proven using a chemically linked anti-CD64 immunotoxin (36); it both developed specific functional activity against CD64 expressing cells in vitro and in a transgenic mice model expressing human CD64.
Although CD64 is a normal marker during the myeloid lineage differentiation pathway, this surface receptor is an ideal target for selective immunotherapy because it is not expressed on CD34-positive hematopoietic stem cells. Self-renewing of these cells is a prerequisite for long-term multilineage reconstitution of hematopoiesis after immunotherapy eliminating human immune effector cells (8). Additionally, it has been shown that only activated CD64-positive cells are killed, whereas CD64-expressing nonactivated cells are not affected (14, 36).

The periplasmically expressed, nonglycosylated recombinant scFv-immunotoxin constructed in this study exhibited specific cytotoxic activity in vitro in the same concentration range (ng/ml) as reported for other ETA-based fusion proteins (28, 37–39).

In summary, we have shown that CD64-positive AML-derived tumor cell lines and primary patient-derived AML cells can be specifically eliminated by a novel recombinant anti-CD64 immunotoxin in vitro. Having demonstrated the functional activity of m22(scFv)-ETA', this selective immunotherapeutic compound might also be used to eliminate deregulated, tissue-infiltrating CD64-positive monocytic/macrophages in patients with local inflammatory diseases (36).

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