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 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 His6-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 10^12 to 10^13 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 10^9 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 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 gyr A46 thi-1 relA1 lac F’ (pro AB’ lacI Q lacZ ΔM15 Tn10 (tet R’))] was used for propagation of plasmids and E. coli BL21 Star (DE3) [FompT hsdS2 (r B g m) gal dcm rna 131 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 Stbl3/Nov2-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 (Qi-
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 (<1.07 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, Tephen, 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 T. Tephen), were cultivated in complete medium (RPMI 1640) supplemented with 10% fetal bovine serum, 50 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine. All cells were cultured at 37°C in a 5% CO₂ air atmosphere.

**Construction and Expression of Recombinant m22(scFv)-ETA.** The m22(scFv) DNA was amplified from a m22-bearing plasmid (provided by T. Tephen) by PCR using the oligonucleotide primers m22(scFv)Back [5’-ATG-GCT-CAG-GTG-GCC-GCC-CAG-CAG-GTG-CCT-GTG-G-3’; bold letters: SfiI consensus site, in italics: 5’-m22(scFv) region] and m22(scFv)For [5’-GAG-TCA- TTC-CTG-AGC-GGC-GG-GCC-CCG-GGC-TTG-CAT-CTC-GCT-GGT-CC-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 pBBM11.22, 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 above (21). Recombinant immunotoxin was eluted with PBS (pH 7.4) and 1 M NaCl, digested with PstI and cleaved with alkaline phosphatase (Sigma Chemical Co., Deisenhofen, Germany) and a solution of Tris-HCl (pH 8.0) and 0.2 mg/ml naphthol-AS-Bi-phosphate (Sigma Chemical Co.) supplemented with 1 mg/ml Fast-Red (Serva, Heidelberg, Germany).

**CM-ELISA.** The binding activity of the fusion protein m22(scFv)-ETA’ was determined by CM-ELISA using biological active membranes of tumor cell lines as described by Tur et al. (26, 27). ELISA Maxisorp-Plates (Nalge Nunc International, Roskilde, Denmark) were coated with 100 µl (0.9 mg protein/ml) freshly prepared membrane fractions of CD64-positive cell lines HL-60/ U-937 and L540Cy as control in 0.02 ml bicarbonate buffer (pH 9.6) overnight at 4°C. Plates were washed five times with PBS (pH 7.4) containing 0.2% (w/v) Tween 20 (TPBS) and blocked with 200 µl 2% BSA (w/v) in PBS (PBSA). After overnight incubation at 4°C, plates were washed five times with TPBS and 2-10 µg/ml m22(scFv)-ETA’ diluted with 0.5% BSA (w/v), with 0.05% Tween 20 (w/v) in PBS at 1 h. Thereafter, plates were washed, and binding of the recombinant immunotoxin was detected with the anti-ETA’ mAb TC-1 and F(ab’)² fragments of peroxidase-coupled goat anti-mouse IgG (Boehringer, Ingelheim, Germany) according to the manufacturer’s recommendations. Bound antibodies were visualized after addition of 100 µl of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) solution (Roche Molecular Biochemicals) by measuring the extinction at 415 nm with an ELISA Reader (Molecular Devices, Ismaning, Germany).

**Flow Cytometric Binding Analyses.** Cell binding activity of m22(scFv)-ETA’ expressed in E. coli BL21 Star (DE3) was evaluated using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Heidelberg, Germany). Cells were stained with the affinity purified scFv-imunotoxin as described previously (28). Briefly, 10,000 events were collected for each sample, and analyses of intact cells were performed using appropriate scatter gates to exclude cellular debris and aggregates. A total of 3 × 10⁶ cells was incubated for 1 h on ice with 50 µl of the m22(scFv)-ETA’ recombinant protein extract at a concentration of 30–40 µg/ml. The cells were washed with PBS buffer containing 0.2% (w/v) BSA and 0.05% (w/v) sodium azide and then incubated for 30 min with an anti-Pseudomonas ETA mAb (TC-1) diluted 1:2 in PBS buffer. Cells were washed and incubated with FITC-labeled goat-anti-mouse IgG (Dako Diagnostika, Hamburg, Germany) for 1 h at 4°C. After a final wash, the cells were treated with 2 µl of 6.25 mg/ml PI and subsequently analyzed by fluorescence-activated cell sorting.

Additionally, as positive control, CD64-positive AML cells were directly identified by a m22(scFv) fragment recombinantly fused to eGFP.

**Colorimetric Cell Proliferation Assay.** The cytotoxic effect of m22(scFv)-ETA’ on target cells was determined by measurement of metabolic zation of XTT to a water soluble orange formazan dye was determined as described previously (28), with the colorimetric assay. The concentration required to achieve a 50% reduction of protein synthesis (IC₅₀) relative to untreated control cells was calculated graphically via Microsoft Excel generated diagrams. All measurements were done in triplicate.

**Flow Cytometric Assay of Apoptosis.** MNCs from patient-blood samples were isolated by Ficoll-Paque centrifugation. Cell-surface CD64 expression was determined by flow cytometry using the m22(scFv)-ETA’ immunotoxin as described above. Additionally, primary leukemic cells were directly stained using the newly developed eGFP-tagged m22(scFv) fusion protein. Approximately 5 × 10⁶ MNCs/well were seeded in flat-bottomed 12-well plates in RPMI 1640 supplemented with 10% FCS in triplicate. A total of 100 ng/ml immunotoxin was added into each well, and the cells cultured for 18 h at 37°C and 5% CO₂ air atmosphere. Apoptotic cells were detected using an annexin V-FITC apoptosis detection kit I (BD PharMingen, Heidelberg, Germany).

**RESULTS**

**Construction and Expression of m22(scFv)-ETA’.** PCR-amplified m22(scFv) DNA (Fig. 1A) was directionally cloned into the kanamycin-resistant pBM11.1 expression vector containing an IPTG-
RECOMBINANT IMMUNOTOXIN FOR THE TREATMENT OF AML

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 ~58% (14). To realize the construction of the immunotoxin, we fused the anti-CD64 scFv m22 to a truncated Pseudomonas exotoxin A (ETA’). The major findings to emerge from our study are: (a) functional m22(scFv)-ETA’ 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’ 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 cytotoxic 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 DT\textsubscript{ss} -GM-CSF combining DT with GM-CSF was evaluated in a Phase I dose-escalation trial in patients with relapsed AML (32). DT\textsubscript{ss} -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 (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 found in hepatic sinusoids (31). The fusion toxin DT\textsubscript{ss} -GM-CSF combining DT with GM-CSF was evaluated in a Phase I dose-escalation trial in patients with relapsed AML (32). DT\textsubscript{ss} -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 (33).

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The most important prerequisite for effective immunotoxin therapy is internalization of target antigen after binding of the immunotoxin to allow 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.

Fig. 3. Binding properties of the recombinant anti-CD64 immunotoxin m22(scFv)-ETA. A, binding of m22(scFv)-ETA to CD64-negative cell membranes L540Cy and CD64-positive AML-derived cell membranes HL-60 documented by CM-ELISA. B, binding of m22(scFv)-ETA to antigen-positive cells by flow cytometry. Cells were stained with purified immunotoxin (transparent curves) or with PBS as negative control (gray curves). C, documentation of specific binding activity of m22(scFv)-ETA using a CM-ELISA with different dilutions of mAb m22 for competition. Binding of m22(scFv)-ETA was detected with peroxidase-conjugated anti-His mAb. Presented are data from three independent experiments.

Fig. 4. Growth inhibition of AML-derived cell lines after incubation with m22(scFv)-ETA as documented by cell-viability assays. A, HL-60 (CD64\textsuperscript{+}) or L540Cy (CD64\textsuperscript{−}) and (B) U937 (CD64\textsuperscript{+}) or IIA1.6 (CD64\textsuperscript{−}) were treated with various dilutions of recombinant anti-CD64 immunotoxin. HL-60, U937 (-----) or L540Cy, IIA1.6 (-----) were treated with m22(scFv)-ETA\textsuperscript{−}, and their ability to metabolize the XTT to a water-soluble formazan salt (formed by mitochondrial dehydrogenase activity) was measured as absorbance at 450 and 650 nm. Measurements were performed in triplicate. Results are presented as percentage of untreated control cells.
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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