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In vitro model of toxin therapy targeted against murine myeloid leukemia cells

Key words G-CSF · *Pseudomonas* exotoxin · Myeloid leukemia

Introduction

The current major obstacles in cancer chemotherapy include acquisition of multidrug resistance and resting cancer cells that are not in the cell cycle. In this paper we discuss one of the candidate strategies for overcoming these problems, focusing on the treatment of myeloid leukemias.

A family of protein toxins is made by plants and bacteria, and these act within the cell cytoplasm to inhibit protein synthesis; *Pseudomonas* exotoxin (PE), diphtheria toxin (DT), and ricin are particularly well known. Toxins and conventional chemotherapeutic agents have different mechanisms of action. Therefore, cancer cells that are naturally resistant or acquire resistance to chemotherapeutic agents will not be cross-resistant to toxin-based therapies. Furthermore, toxins are potentially cytotoxic for nondividing cells that cannot be killed by conventional drugs. These unique properties make them attractive for use in the treatment of cancer. For this purpose, PE, DT, and ricin have been chemically or genetically attached to monoclonal antibodies and polypeptide hormones to direct their cytotoxic activities to specific eukaryotic cells [14, 15].

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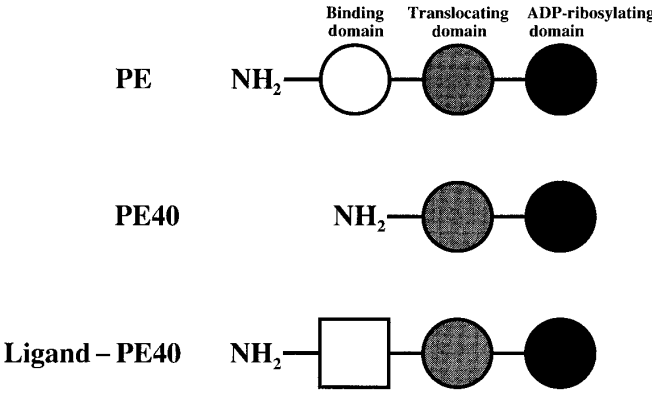

Fig. 1 Targeting of PE to receptors and antigens. PE40, which lacks the binding domain (domain *Ia*), can be expressed in *Escherichia coli* as a fusion protein with an appropriate ligand (*ligand-PE40*). This fusion protein can then target specific receptors or antigens

Pseudomonas exotoxin

PE consists of three structural domains [1]: domain Ia binds to the cell surface receptor, domain II acts in translocation across the cell membrane into the cytosol, and domain III is involved in adenosine diphosphate (ADP) ribosylation of elongation factor 2, the process essential for cell death [5]. No role for domain Ib has been determined [17]; a 40-kDa mutant of PE that lacks domain Ia (PE40) retains full enzymatic activity but is not cytotoxic for cells because endocytosis does not occur. The cytotoxic activity of PE40 can be restored by fusion of the gene segment encoding PE40 to cDNAs encoding growth factors including transforming growth factor-α (TGF-α) [2], interleukin 2 (IL-2) [7], and IL-6 [16], and expression of these chimeric genes in *Escherichia coli* (Fig. 1).

Candidate ligands for leukemia therapy

Candidate ligands for leukemia therapy are shown in Table 1 and include hematopoietic growth factors, ILs, and

Table 1 Candidate ligands for leukemia therapy

Ligand type	Examples
Hematopoietic growth factor	G-CSF, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, stem-cell factor, FLK2 ligand
IL	IL-2, -3, -4, -6, -7…
Monoclonal antibodies	Anti-CD4, -CD7, -CD10, -CD25, -CD33, -CD34, -P-glycoprotein

monoclonal antibodies against surface antigens expressed on hematopoietic cells. We selected granulocyte colony-stimulating factor (G-CSF) as a ligand for targeting myeloid leukemias because it is highly specific for myeloid cells and lacks significant effects on other tissues. G-CSF is a 20-kDa glycoprotein produced by activated macrophages, endothelial cells, and fibroblasts. G-CSF primarily regulates production of, enhances maturation of, and activates neutrophilic granulocytes [19]. Injection of healthy mice with pharmacologic doses of G-CSF results in a marked increase in granulocyte and committed progenitor numbers in the absence of significant hematopoietic effects on other blood-cell types [3]. Furthermore, mice lacking G-CSF show chronic neutropenia, myeloid progenitor-cell deficiency, and impaired neutrophil mobilization [6]. G-CSF also stimulates clonogenic growth of myeloid leukemia cells in vitro [9, 20]. In accordance with this, G-CSF specifically binds to a cell-surface receptor that can be detected only on mature neutrophils and their progenitors, except in the placenta, as well as a majority of myeloid leukemia cells [4, 10, 13]. Therefore, G-CSF appears to be an attractive vehicle for delivering biological substances not only to myeloid progenitors but also to myeloid leukemia cells.

Construction and expression of G-CSF–PE40

The plasmid pGL411 was expressed under the control of the phage T7 late promoter [11]. The fusion protein produced, G-CSF–PE40, consists of an amino-terminal methionine residue and amino acids 1–174 of mature human (h) G-CSF fused to amino acids 253–613 of PE via a 3-amino-acid linker, lysine-alanine-phenylalanine. *E. coli* BL21 (λDE3) was transformed with pGL411, and production of the hybrid toxin was induced using β-D-thiogalactoside (IPTG). Immunoblot analysis using both anti-G-CSF and anti-PE antibodies revealed the presence of an IPTG-inducible protein migrating with the expected molecular mass of G-CSF–PE40. The hybrid toxin was purified by sequential chromatography.

G-CSF receptor binding

To test whether G-CSF–PE40 would bind to the G-CSF receptor, a competitive binding assay was performed on peripheral blood neutrophils, and it was shown that G-CSF–PE40 competes with G-CSF binding dose-dependently [11]. To examine the biological activity of G-CSF–PE40 we used NFS60 cells passaged in the presence of recombinant hG-CSF (rhG-CSF). Of the many murine factor-dependent hematopoietic cell lines, only this line can grow in the presence of either IL-3 or G-CSF. However, NFS60 cells passaged in the presence of G-CSF show a markedly reduced response to IL-3, and vice versa. Therefore, in the presence of 100 p*M* rhG-CSF, NFS60 cells were incubated with serial 2-fold dilutions of G-CSF–PE40. Protein synthesis after 48 h of incubation was inhibited by G-CSF–PE40 dose-dependently [11]. The 50% inhibitory dose (ID_{50}) was estimated to be approximately 100 p*M* under these assay conditions.

Cytotoxic activity of G-CSF–PE40

We examined the cytotoxic activity of G-CSF–PE40 using the rapid colorimetric assay, in which only viable cells can produce colored formazan by reducing 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazorium (MTT). After a 48-h incubation period, G-CSF–PE40 exhibited dose-dependent cytotoxicity for NFS60 cells [11]. G-CSF–PE40 was not active on Ba/F3 cells, which do not express G-CSF receptors.

Mitogenic signal transduction by G-CSF–PE40

The question as to whether G-CSF–PE40 could transduce mitogenic signals was also tested. After 16 h, G-CSF stimulation of [^{3}H]-thymidine uptake was inhibited by the hybrid toxin only at high concentrations. In contrast, G-CSF–PE40 alone stimulated [^{3}H]-thymidine uptake at lower concentrations, indicating that G-CSF–PE40 initially acts as a G-CSF agonist [11]. This result suggests that G-CSF–PE40 may exert potent cyotoxicity in combination with S-phase-specific drugs such as cytosine arabinoside (Ara-C). However, as G-CSF–PE40 cannot discriminate between normal and leukemic myeloid cells, we consider that it may be used as a conditioning agent in bone marrow (BMT) or peripheral blood stem-cell transplantation.

Discussion

Although recent advances in leukemia therapy have improved the overall prognosis of patients, some patients have refractory leukemias that exhibit high relapse rates even after BMT. Two major problems exist in treatment of these leukemias. First, leukemic cells are naturally resistant or acquire resistance to chemotherapeutic agents. Second, a significant number of leukemic cells are nondividing, dormant cells [8] that are insensitive to chemotherapy. Toxins have mechanisms of action different from those of

conventional chemotherapeutic agents and can kill nondividing cells [13, 15]. These common properties of toxins make them attractive for use in leukemia therapy.

Targeting PE to G-CSF receptors results in elimination of myeloid leukmia cells and normal myeloid progenitor cells. Nevertheless, G-CSF–PE40 is an attractive agent in conditioning regimens for BMT, in which healthy stem/progenitor cells are transplanted into patients just after total eradication of hematopoietic cells. The neutralizing antitoxin response, usually evoked within 2 weeks of injection of chimeric toxins, is negligible in such cases [12]. Our present conditioning regimen for refractory myeloid leukemias includes total-body irradiation followed by high-dose Ara-C with continuous infusion of G-CSF [18]. This strategy is based on experimental results showing that G-CSF increases the susceptibility of leukemic cells to Ara-C by enhancing their entry into the S phase [18]. From this point of view, use of G-CSF–PE40 appears more beneficial because it may initially act as a G-CSF agonist and later exhibit cytotoxicity for target cells.

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