ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 33 (2005) 767–775

RNAi-mediated knockdown of P-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines

Holger Rumpold[a], Anna M. Wolf[b], Kurt Gruenewald[a], Guenther Gastl[a], Eberhard Gunsilius[a,*], and Dominik Wolf[a,*]

[a]*Laboratory of Tumorbiology and Angiogenesis, Division of Hematology and Oncology and*
[b]*Division of Gastroenterology and Hepatology, Medical University of Innsbruck, Innsbruck, Austria*

(Received 1 November 2004; revised 9 March 2005; accepted 23 March 2005)

***Objective*. Resistance to therapeutic drugs is a frequent phenomenon in hematologic malignancies, causing treatment failure in patients with leukemias and lymphomas. Overexpression of the multidrug-resistance gene (MDR-1) and its translational product P-glycoprotein (PgP) represents one mechanism of fatal drug resistance.**

***Methods*. We constructed a nonviral, transposon-based vector system for the stable knockdown of PgP in chronic myeloid leukemia cell lines resistant to imatinib and doxorubicin.**

***Results*. Using this strategy, PgP expression was completely knocked down 72 hours after vector inoculation and lasted for several months. Cellular efflux of the PgP substates rhodamine and doxorubicin was abolished. Vector-treated cells were resensitized to imatinib- and doxorubicin-induced cell death.**

***Conclusion*. Using chronic myeloid leukemia as a model, we show that PgP-mediated resistance to imatinib and anthracyclines can be durably reversed by nonviral, transposon-based knockdown of PgP in malignant cells. © 2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

Introduction

Resistance to cytotoxic drugs represents a serious hindrance in cancer chemotherapy. Inhibitors of growth factor signaling, such as antibodies and small molecules, are promising candidates that might be active against malignant cells even if they are resistant to conventional cytotoxic drugs. However, innate or acquired resistance to these novel substances can also occur. One such small molecule is imatinib (Glivec), a potent inhibitor of the bcr/abl kinase, which blocks its ATP-binding pocket [1]. Treatment with imatinib has been shown to be very effective in chronic myeloid leukemia (CML) [2–4]. However, a significant number of patients, especially with advanced disease, develop resistance to imatinib [5]. Imatinib resistance can be mediated by bcr/abl gene amplification and mutations, but also by the overexpression of P-glycoprotein (PgP) in leukemic cells [6–8].

The silencing of the multidrug-resistance gene (MDR-1), resulting in diminished PgP expression, represents an attractive strategy to revert drug resistance. Several strategies to silence MDR-1 in malignant cells have been investigated, such as oligonucleotides [9], hammerhead ribozymes [10,11], and transient siRNA technologies [12,13]. However, the long half-life of PgP of at least 16 hours is a severe problem to achieve the complete knockdown of PgP [14]. To circumvent this problem, siRNA vectors have been adapted to retro-, adeno-, and lentiviral systems. Virus-associated difficulties, such as insertional mutagenesis, cis-activation of silent genes by their strong promoters, and the difficult generation of recombinant viruses restrict the use of such systems [15–17].

An alternative and promising new strategy is the transposon-based Sleeping Beauty system (SB). SB is a member of the Tc1/mariner superfamily of transposable elements, and a synthetic transposase which regained activity in human cells has been developed [18,19]. This system induces a preferential insertion of the transposon into TA-rich sites of the host genome [20] and is active in a variety of cell types [21,22]. The SB system is the only non-viral DNA-transposon

Offprint requests to: Eberhard Gunsilius, M.D., Laboratory of Tumorbiology & Angiogenesis, Division of Hematology and Oncology, Medical University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria; E-mail: eberhard.gunsilius@uibk.ac.at

*These authors contributed equally to this work.

0301-472X/05 $–see front matter. Copyright © 2005 International Society for Experimental Hematology. Published by Elsevier Inc.
doi: 10.1016/j.exphem.2005.03.014

active system. Here, we describe the generation of an SB-based RNAi system for the stable knockdown of MDR-1, restoring sensitivity to imatinib in PgP-overexpressing, imatinib-resistant chronic myeloid leukemia cells. This vector system represents a powerful tool for the circumvention of PgP-mediated drug resistance in malignant cells.

Materials and methods

Cell culture

PgP-overexpressing K562/Dox cells (a kind gift of J. P. Marie, INSERM, E9912, University of Paris 6, France) were obtained by long-term culture in the presence of doxorubicin. Cells were cultured in RPMI 1640 containing 2% glutamine, penicillin, streptomycin (Invitrogen, Lofer, Austria), 10% fetal calf serum (FCS; Invitrogen, Lofer, Austria), and 1 nM doxorubicin at a density of 2×10^5 and were subcultured upon reaching a density of 1×10^6 cells per mL. One week before nucleofection doxorubicin was omitted from the medium.

Vector construction

The neomycin (neo) resistance cassette was obtained as an EcoRI-SalI fragment from pMGD20-Neo (a kind gift from M. Gassmann, ETH Zurich, Zurich, Switzerland). The ends of the fragment were filled in by Klenow-enzyme and ligated into the blunt-ended HindIII site of pT2-HB (a kind gift of P. Hackett, University of Minnesota, Minneapolis, MN, USA), thereby obtaining pT2Neo. The H1-promoter was PCR-amplified from pEPU-H1 (a kind gift of S. Geley, Innsbruck, Austria), inserting a NotI site upstream and restriction sites NheI followed by BamHI downstream of the H1-promoter. This fragment was NotI/BamHI-digested and inserted into the NotI-BglII-digested pT2Neo, destroying the downstream BglII site by ligating to the fragment's BamHI site, rendering the vector pT2Neo-H1 (Fig. 1A). Chemically synthesized (MWG-Biotech, Munich, Germany) oligonucleotides coding for siRNA were inserted into the BglII-NheI linearized vector fragment. One oligonucleotide (siMDR1/A), targeting the sequence 5′-AGAAACCAACTGT-CAGTGTAT-3′ located at +67 to +87 bp in the coding sequence of MDR1 (GenBank #M14758), was found to be most efficient. Vectors used for knockdown experiments were: pT2Neo-H1 as the empty vector control; pT2Neo-A1 carrying a nonmutated siRNA encoding cDNA-oligonucleotide targeting the MDR1 mRNA at the site mentioned above; pT2Neo-MM carrying the same siRNA encoding cDNA-oligonucleotide with a mismatch in its sense-strand for destabilization of the siRNA hairpin at its 5′-end (sequence: 5′-AGAAACCAACTGTCAGTGT**G**T-3′; fat guanine indicates the mismatch nucleotide). The vector pT2Neo-Co was used as a specificity control containing a siRNA-oligonucleotide with three-bp mutation in the center of the siRNA antisense strand to destabilize the double-strand formation between the siRNA and the MDR1 mRNA (sequence: 5′-AGAAACCAA**GAC**TCAGTGTGT-3′; fat and underlined indicates the mutation). The linker sequence for all hairpins was 5′-TTCAAGAGA-3′. The helper plasmid coding for the Sleeping Beauty transposase was a kind gift of P. Hackett, University of Minnesota (Fig. 1B). For control purposes a transposon-based vector containing EGFP driven by an EF1α-promoter was constructed by inserting the EF1α-promoter-EGFP-pA cassette into the NotI-BglII fragment of pT2Neo (Fig. 1C), designated pT2Neo-EGFP.

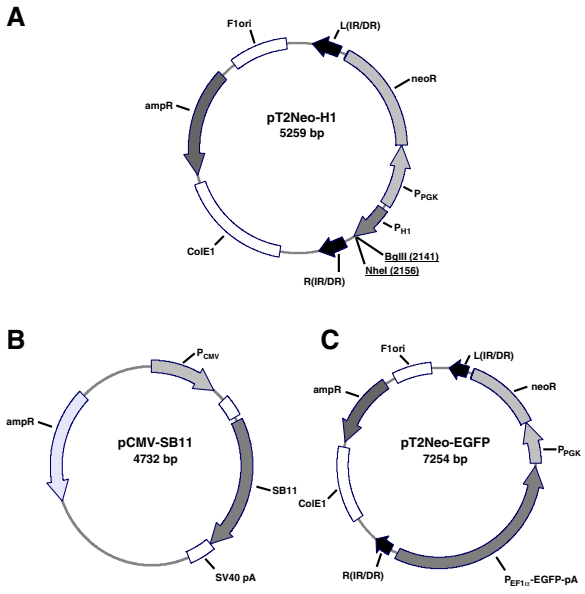

Figure 1. SB-based vectors for RNAi. pT2Neo-H1 contains a neo-resistance gene and the H1-promoter. The cDNA oligonucleotide coding for the siRNA was inserted between the BglII and NheI sites (**A**). For target sequence see material and methods. Helper plasmid coding for SB transposase (**B**) and pT2Neo-EGFP encoding an EGFP construct in the transposon for control purposes (**C**).

Nucleofection and selection of K562/Dox cells and derivatives

A total of 1.5×10^6 cells were nucleofected with the Cell Line Nucleofector Kit-V (Amaxa, Köln, Germany) strictly according to the instructions of the manufacturer. Nucleofection efficiency was approximately 70%. To obtain stable transfected cell lines, the cells were selected with 1 mg/mL neo 24 hours after nucleofection for 2 months. Purity of this cell population was determined by FACS analysis of K562/Dox nucleofected with pT2Neo-EGFP, designated K562/Dox-EGFP and established in parallel to the other cell lines. Control cells nucleofected with pT2Neo-H1 were designated K562/Dox-H1, cells nucleofected with pT2Neo-A1 were designated K562/Dox-A1, K562/Dox-MM were nucleofected with pT2Neo-MM, and K562/Dox-Co were nucleofected with pT2Neo-Co.

Real-time PCR and PCR on genomic DNA

Real-time PCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Briefly, 500 ng of isolated total RNA was reverse-transcribed and analyzed for MDR1 mRNA by employing sense primer 5′-aaacaccactggag-cattgactac-3′, antisense primer 5′-ctggaacctatagccccctttaa-3′, and probe 5′-FAM-aggctcgccaatgatgctgctcaa-TAMRA-3′. β2 microglobulin (β2MG) was used as housekeeping gene and obtained as a kit (Applied Biosystems). Efficiency was calculated as the average slope of a cDNA-dilution row of three independent runs and was 1.90 for MDR and 1.81 for β2MG. Repression of MDR1 was calculated according to the equation formulated by Pfaffl et al. [23].

For detection of the SB-transposon two primer sets consisting of the sense primer 5′-tatccatggaattcactagtgcg-3′ located closely upstream of the H1-promoter in pT2Neo-H1, the antisense primer for K562/Dox-A1 5′-tctcttgaaatacactgacagttggt-3′ and for K562/Dox-MM 5′-tctcttgaaacacactgacagttggt-3′ enclosing the linker and its flanking region of the respective hairpin were used. PCR was performed with 1 μg genomic DNA of the indicated cell lines,

Download English Version:

https://daneshyari.com/en/article/10908092

Download Persian Version:

https://daneshyari.com/article/10908092

Daneshyari.com