Specific inhibition of bcr-abl gene expression by small interfering RNA

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Abstract

Small interfering RNAs (siRNA) were designed to target the bcr-abl oncogene which causes chronic myeloid and bcr-abl positive acute lymphoblastic leukemia (CML, ALL). Chemically synthesized anti-bcr-abl siRNAs were selected using reporter gene constructs and were found to reduce bcr-abl mRNA up to 87% in bcr-abl positive cell lines and in primary cells from CML patients. This mRNA reduction was specific for bcr-abl since both c-abl and c-bcr mRNA levels remained unaffected. Furthermore, protein expression of BCR-ABL and of laminA/C was reduced by specific siRNAs up to 80% in bcr-abl positive and normal CD34+ cells, respectively. Finally, anti-bcr-abl siRNA inhibited BCR-ABL-, but not cytokine-dependent proliferation in a bcr-abl positive cell line. These data demonstrate that siRNA can specifically and efficiently interfere with the expression of an oncogenic fusion gene in hematopoietic cells.

Introduction

RNA interference (RNAi) describes a highly conserved regulatory mechanism that mediates sequence-specific post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA) (1-3). The RNase III enzyme Dicer processes dsRNA into ~22 nucleotides (nt) small interfering RNAs (siRNA) (4) which serve as guide sequences to induce target-specific mRNA cleavage by the RNA-induced silencing complex RISC (5). In plants and C. elegans, RNAi may involve the amplification of dsRNA by a RNA-dependent RNA polymerase (RdRP) (6) and enables systemic, long term, and heritable gene silencing. In contrast, RNAi in Drosophila and mammals seems cell autonomous, transient and non-heritable. Since exogenous application of siRNAs can efficiently trigger RNAi in mammalian cells (7,8), siRNAs
are increasingly used in transient (co)transfection assays to modulate gene expression in mammalian including human cells (9-12).

Fusion-transcripts encoding oncogenic proteins may represent potential targets for a tumor-specific RNAi-approach. The Philadelphia (Ph-) translocation t(9;22)(q34;q11) generates the bcr-abl fusion gene characteristic for chronic myelogenous leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL) (13). Bcr-abl encodes a constitutively active cytoplasmic tyrosine kinase that is both necessary and sufficient to induce and maintain leukemic transformation (14-16).

We demonstrate that anti-bcr-abl siRNAs specifically inhibit bcr-abl mRNA expression in hematopoietic cell lines and primary CML cells. They reduce BCR-ABL protein expression and inhibit BCR-ABL, but not cytokine-dependent cell proliferation. Therefore, anti-bcr-abl siRNAs may allow further analysis of BCR-ABL functions, and, eventually, may lead to RNAi-based therapeutics.

**Material and Methods**

*siRNAs*

21-nt single-stranded RNAs directed against the fusion sequence of bcr-abl were chemically synthesized (BioSpring, Frankfurt, Germany)(Figure 1A). The sense and antisense sequences were b3a2_1: 5’-GCAGAGUUCAAAGCCCUUdTdT-3’, b3a2_3: 5’-AGCAGAGUUCAAAGCCCUUdTdT-3’, and b3a2_1: 5’-AAGGGCUUUUGAACUCUGCdTdT-3’, b3a2_3: 5’-AGGGCUUUUGAACUCUGCdTdT-3’, respectively. Control anti-GL2_ and invGL2_siRNAs targeting GL2-luciferase, anti-laminA/C siRNAs, and siRNA-duplexes were generated as described by Elbashir et al. (7).
Transfection of hematopoietic cells

K562, murine TonB cells derived from the BaF3 cell line, and primary hematopoietic cells were cultured as described (17-19). 1x10⁶ cells each were electroporated (330V, 10mA) in 100µl of RPMI 1640/10%FCS containing 0.5 µg siRNA duplex in a 4 mm electroporation cuvette using an EPI2005 gene pulser (Fischer, Heidelberg, Germany).

Real-time RT-PCR

Real-time Taqman-RT-PCR was performed as described (20,21) with the probes and primers: bcrFP: 5’-AGCACGGACAGACTCATGGG - 3’, bcrRP: 5’-GCTGCCAGTCTCTGCTCTGC-3’, bcr-Taqman-probe: 5’-AGGGCCAGGTCCAGCTGGACCC-3’ covering the exon b5/b6 boundary, ablFP: 5’-GGCTGTCTCAGCTCTCCAG-3’, ablRP: 5’-TCAGACCCTGAGGCTCAAAGT-3’, abl-Taqman-probe: 5’-ATCTGGAAGAAGCCCTTCAGCGGC-3’ covering the exon 1a/2 boundary. RNA from murine TonB cells was digested with DNAsel and analysed using the probe and primers: GAPDHmu5’: 5’-CAACAGGGTTGGTGACCT-3’, GAPDHmu3’: 5’-GGGTGGTCCAGGGTTTCTTA-3’, and GAPDHmu-Taqman-probe: 5’-TGGCCTACATGGCCTCAAAGGA-3’.

Immunoblotting and immunofluorescence microscopy

Cellular lysates from TonB cells were immunoblotted with polyclonal anti-bcr antibody (N-20) and polyclonal anti-Stat5 antibody (C-17) as described (18). To quantify transduction efficacy, laminA/C immunostaining was performed using a monoclonal anti-laminA/C antibody (sc-6215, all antibodies from Santa Cruz Biotechnology) as described (7).
Primary normal and CML cells

Normal and CML CD34+ cells were purified to ≥ 95% as described (19). Primary CD34+ or peripheral blood derived mononuclear cells (PBMNC) cells were cultured in X-VIVO/1% HSA with recombinant human SCF (100 ng/ml), Flt-3-ligand (100 ng/ml), and TPO (20 ng/ml) before electroporation, and GM-CSF and IL-3 (10 ng/ml each) were added thereafter. Methylcellulose colony assays were performed as described (19).

Results and Discussion

Selection of anti-bcr-abl siRNAs

Efficient siRNAs targeted against the b3a2-fusion sequence of bcr-abl were selected by cotransfection with a chimeric bcr-abl-EGFP reporter gene in HeLa cells. Hela cells could be transfected up to 95% (data not shown). Out of four chemically synthesized 21-nt siRNAs tested, b3a2_1 and b3a2_3 (Figure 1A) were the most efficient siRNAs. They reduced the number of fluorescent cells (up to 90%), bcr-abl-EGFP mRNA levels (up to 87%), and the fluorescence intensity per cell (up to one-hundred-fold) 24 hours after transfection (data not shown). RNAi was specific since no reduction in fluorescence intensity was found with control siRNAs or when native EGFP without bcr-abl sequences was used as reporter. Furthermore, anti b3a2-bcr-abl siRNA only reduced the b3a2-, but not the b2a2- variant of a bcr-abl-EGFP reporter gene and vice versa (data not shown).

Effects of siRNAs in hematopoietic cell lines

b3a2_1 and b3a2_3 siRNAs were tested in Ph+ K562 and TonB cells expressing bcr-abl under control of a doxycycline inducible promoter (17).
Transfection efficacy was analysed using the laminA/C system and reached about 80% in K562 cells (data not shown). b3a2_1 and b3a2_3, but not control siRNAs, reduced bcr-abl mRNA levels after 24 and 48 hours up to 24.8% and 35.2% (b3a2_1), and 32.4% and 61% (b3a2_3), respectively (Table 1 and data not shown). Notably, c-bcr and c-abl mRNA levels remained unaffected demonstrating the specificity of anti-bcr-abl siRNAs (Table 1). Furthermore, b3a2_1 and b3a2_3 transiently reduced the number of viable K562 cells by 75% in suspension cultures 4 days after electroporation (data not shown).

Cultures of TonB cells with or without doxycycline and IL-3 allow separate studies of BCR-ABL- and IL-3-mediated cell proliferation with factor-independent proliferation considered as a surrogate marker for cellular transformation. After electroporation with b3a2_1 siRNA, bcr-abl mRNA declined by about 70% independent of IL-3 (Figure 1B). Furthermore, b3a2_1 siRNA reduced BCR-ABL protein expression by about 55% as analysed by immunoblotting and densitometry (Figure 1C). Finally, b3a2_1 siRNA reduced the number of viable TonB cells to a similar extent as the selective tyrosine kinase inhibitor STI571 in the absence but not in the presence of IL-3 (Figure 1D,E). Again, cultures of TonB cells treated with b3a2_1 resumed growth after 3 to 5 days in all conditions tested. In parental bcr-abl-negative BaF3 cells siRNA had no effect on cell proliferation (data not shown).

siRNA activity in primary hematopoietic cells

siRNA efficacy in primary hematopoietic cells was first demonstrated in normal CD34+ cells using the laminA/C system. Anti-laminA/C siRNA reduced laminA/C protein expression by about 70% (Figure 1F). Next, electroporation of PBMNCC or purified CD34+ from six CML patients with b3a2_1 siRNA reduced bcr-abl-mRNA levels by 50% to 79% in comparison to control siRNA (100%) (Table 1). Again, c-bcr
and c-abl mRNA levels remained unchanged in both CML samples studied. When primary CML cells were transfected with b3a2_1 siRNA and grown in cytokine supplemented liquid or semisolid cultures, no significant inhibition of cell proliferation or colony formation was observed (Table 1 and data not shown). In contrast, STI571 markedly reduced the number of viable cells in suspension cultures and the colony number derived from purified CD34+ cells as shown in earlier studies (22) (Table 1 and data not shown).

Our data show gene suppression mediated by siRNA in normal and malignant hematopoietic cells. Specifically, siRNAs induced a specific but transient reduction of bcr-abl mRNA and protein expression, and inhibition of BCR-ABL mediated cell proliferation. As expected for mammalian cells (2,3,23) we found no evidence for transitive RNAi involving RdRP since anti-bcr-abl siRNAs did not affect c-bcr and c-abl mRNA levels. The markedly different effects of anti-bcr-abl siRNA and STI571 on CML cells in our study may be explained by the transient and non-heritable nature of RNAi in mammalian cells and the protein half-life of BCR-ABL. Alternatively, inhibition of bcr-abl expression by siRNA or antisense sequences (24) as compared to blocking BCR-ABL kinase activity by STI571 may induce different phenotypes in cytokine supplemented cultures of bcr-abl positive cells (22,24). The molecular basis for these differences, and the kinetics of RNAi triggered by exogenous or endogenous expression of siRNAs (10,25) should be analyzed to better define the role of RNAi as scientific tools or potential therapeutics in human hematopoietic cells.

Acknowledgements

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References


Figure legend

Fig. 1. RNA interference in hematopoietic cells

(A) Bcr-abl fusion sequence and schematic representation of b3a2_1 and b3a2_3 siRNAs shifted by only one nucleotide. TT stands for the deoxythymidine dimer as 3´
overhang. The arrow marks the fusion between bcr- (left) and abl- (right) sequences of the b3a2-bcr-abl variant.

(B) siRNA mediated reduction of bcr-abl mRNA expression in TonB cells in the presence or absence of IL-3. Normalized bcr-abl/GAPDH mRNA levels were measured 24 h after electroporation and are shown in comparison to control cells treated with GL2/invGL2 control siRNAs (100%). The data represent mean values and standard deviations from three independent experiments.

(C) Immunoblot of TonB cells treated with doxycycline and siRNAs. TonB cells were induced to express BCR-ABL (lanes 1, 3-8) or not (lane 2) by addition of doxycycline at 1 µg/ml. Cells were electroporated with b3a2_1 siRNA (lanes 4,6, and 8) or control GL2_siRNA (lanes 3,5 and 7) and lysed 8 (lanes 3 and 4), 24 (lanes 5 and 6), and 60 hours (lanes 7 and 8) after electroporation, respectively. The upper panel shows an immunoblot with anti-BCR-specific antibodies, and the lower panel the same membrane reprobed with anti-Stat5 antibodies as loading control.

(D and E) Effects of siRNAs on BCR-ABL (D) and IL-3 (E) mediated cell proliferation. TonB cells were either electroporated with control GL2_ (red squares) or b3a2_1 (blue filled circles) siRNA or left untreated in cultures containing 1 µM of STI571 (orange empty circles). Viable cells were counted by trypan blue exclusion during suspension cultures after addition of doxycycline at 1 µg/ml without (D) or with (E) murine IL-3. Cell numbers of b3a2_1 and STI571 treated cells were nearly identical in the presence or absence of IL-3.

(F) Inhibition of laminA/C protein expression by siRNAs in normal CD34+ cells. Normal CD34+ cells were electroporated with control GL2_ (left panels) or anti-laminA/C siRNAs (right panels). The upper panels show immunostaining of laminA/C and the lower panels nuclear chromatin staining with DAPI.
A

bcr-abl (b3a2-variant)

5'...UGGAUUUAAGCAGAGUUCAA AAGCCCUUCAGCGGCCAGUA...3'

b3a2_1

TT  N_{19}  TT

b3a2_3

TT  N_{19}  TT

B

C

D

E

F

GL2_siRNA

LaminA/C_siRNA

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Table 1 Effects of anti-bcr-abl siRNAs on bcr-abl mRNA expression and colony formation of primary CML cells

<table>
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<th>bcr-abl / GAPDH [%]</th>
<th>c-bcr / GAPDH [%]</th>
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Colony Assay

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The RNA data represent normalized bcr-abl, c-bcr, and c-abl values from K562, primary PBMC and purified CD34+ CML cells 24 hours after electroporation with the respective siRNAs. Controls for each patient were set 100%. K562 data represent mean values and standard deviations from four independent experiments. Standard colony assays were performed with PBMC (#1-4) after electroporation with the respective siRNA. Purified CD34+ cells from #5 were first cultured in standard colony assays and the samples “No STI571” and “1µM STI571” were not electroporated. The last four lanes show CD34+ cells (#5, #6) first grown in suspension culture for 3 days and then plated into standard colony assays. No viable cells were found after suspension culture in the presence of 1µM STI571 after 3 days (#5). nd – not done.
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