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ORIGINAL PAPER

Bcl-2 Antisense Oligonucleotide Inhibits the Proliferation of Childhood Leukemia/lymphoma Cells of the B-cell Lineage

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Abstract An 18-mer phosphorothioate bcl-2 atisense oligonucleotide (ASO) inhibited colony formation of three B-cell leukemia/lymphoma cell lines in a dose dependent manner in the range of 0.125–0.5 μmol/l. The srcambled cogener had no detectable effect. A decrease in BCL-2 protein and apoptotic DNA fragmentation was detected in the studied cell lines and primary blast cells of two children with acute lymphoblastic leukemia. Neither BCL-2 protein level, nor DNA integrity was affected by the scrambled control indicating the specific effect ASO. As far as we know, this is the first report on the effects of bcl-2 ASO on childhood leukemia/lymphoma cell samples.

Keywords BCL-2 · Antisense oligonucleotide · Childhood · Leukemia · Lymphoma

Introduction

Antisense oligonucleotides (ASO), short single stranded DNA molecules composed of 15-25 nucleotides complementary to a key region of mRNA transcribed from target genes have recently been introduced in the armamentary of anticancer treatment [1]. An attractive target of ASO therapy is the bcl-2 gene, the first identified negative regulator of apoptosis [2]. In addition to centrocytic-centroblastic non-Hodgkin lymphoma, increased expression of bcl-2 has been found in a variety of human tumors, including acute myeloid leukemia (AML), melanoma, lung, colorectal and breast cancer [3, 4]. A bcl-2 antisense oligonucleotide oblimersen has been developed by Genta (Genta International Inc, Berkeley Heights, NJ, USA). Genta has reported results from randomised phase III trials of oblimersen in four different indications: malignant melanoma, chronic lymphocytic leukemia, multiple myeloma and acute myeloid leukemia [5].

In this study we investigated the effect of an 18-mer antisense bcl-2 oligonucleotide in leukemia/lymphoma cell lines and in primary blast cells of children with acute lymphoblastic leukemia (ALL) of the B cell lineage. The sequence was designed against bcl-2 mRNA [6]. To increase the resistance against nucleases the phosphodiester internucleotide linkages were converted to phosphorothioate linkages replacing one of the non-bridging oxygen atoms by sulfur [2]. The interaction of the bcl-2 ASO with the bcl-2 mRNA has been shown to inhibit BCL-2 protein expression in cancer cells, thereby enhancing their death by propensity toward apoptosis.

Materials and Methods

Cell Lines

JY is an EBV genom-positive, B lymphoma cell line [7]. BL-41 is an EBV genom-negative Burkitt lymphoma cell line and BCBL-1, a HHV-8-positive but EBV- and HIV-1-negative body cavity based lymphoma cell line [8, 9]. The cells were maintained at 37°C in a humidified athmosphere (5% CO_2) in RPMI-1640 supplemented with 10% FBS, 1% L-glutamin and antibiotics. The cell cultures were split every 2–4 days,

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Table 1 Characterization of two children with acute lymphoblastic leukemia

Patient	FAB type	Immunophenotype	Cytogenetics/DNA index	Molecular genetics
No. 1, 5-year-old girl	L1	CD19+, CD10+ CD34+, CyIgM-, CD33-TdT+	Karyotyping: unsuccessful DNA index: 1.02	Monoclonal rearrangements in theTcRgamma and IgH genes
No. 2, 10-year-old boy	L1	CD19+, CD10+CD34+, CyIgM-, CD33+ TdT+	52,XY,+17,+18,+21,+21,+22, +mar/46,XY DNA index: 1.1	Monoclonal rearrangement in the IgH gene

depending on the cell lines, and harvested in the logarhitmic phase of growth for the experiments.

Patients

Primary leukemic lymphoblasts of a 5-year old girl and a 10-year old boy (Caucasians, both) were studied. Diagnosis was established according to FAB criteria, immunophenotyping, karyotyping and molecular genetics (Table 1).

Primary Leukemic Cells

Excess diagnostic bone marrow sample was obtained with informed consent by iliac crest aspiration. The mononuclear cell (MNC) fraction was separated by Ficoll-Iodamide (specific gravity 1.077 g/ml; Pharmacia, Uppsala, Sweden) gradient centrifugation. The MNC suspension contained >95% leukemic lymphoblasts as checked on May-Grünwald-Giemsa-stained cytospin slides.

Synthesis of Oligonucleotides

Phosphorothioated bcl-2 ASO corresponded to the first 18 bases of the translation start site of bcl-2 gene with the following sequence: TCTCCCAGCGTGCGCCAT [6]. Scrambled oligonucleotide (SCR-ON) was designed in our laboratory, having the following sequence CGACCTAGGCCTTCTGCC. The oligonucleotides were synthesized by standard phosphoramidite chemistry on a Pharmacia Gene Assembler Plus oligonucleotide synthesizer. The phosphorothioate internucleotide linkages were introduced by using Beaucage reagents as thioating agent [10]. The purification was performed on an ÄKTA® PURIFIER (Amersham-Pharmacia) as described [11].

Colony Assay

Clonal growth was assessed in vitro in semisolid methylcellulose culture as described [12]. Cultures containing 0.125–5.0 μmol/l concentrations of either ASO or SCR-ON were plated in triplicates. Control plates did not contain oligonucleotides. Colonies, defined as groups of at least 50 cells were counted under the dissecting microscope (SZ6045; Olympus, Hamburg, Germany) at the end of the incubation period. Results were expressed as relative plating efficiencies in % of controls. I.e. the drug concentration reqiured to kill 50% of the leukemia cells was calculated using the formula: ([% leukemic cell survival (LCS) > 50%] − 50 / ([% LCS > 50%] − [LCS < 50%]) * (drug concentration above 50% LCS − drug concentration below 50% LCS) + (drug contcentration below LCS 50%). [13].

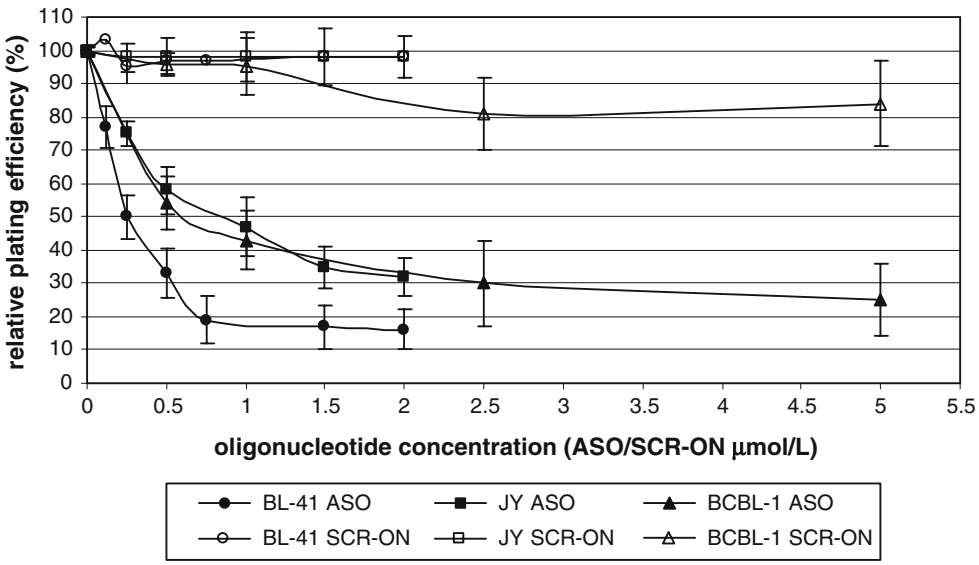

Fig. 1 bcl-2 antisense oligonucleotide (*ASO*) inhibited clonal proliferation of leukemia/lymphoma cell lines In contrast to ASO, scrambled oligonucleotide (*SCR-ON*) did not inffluence clonal proliferation of leukemia/lymphoma cell lines. *Bars* indicate standard deviations

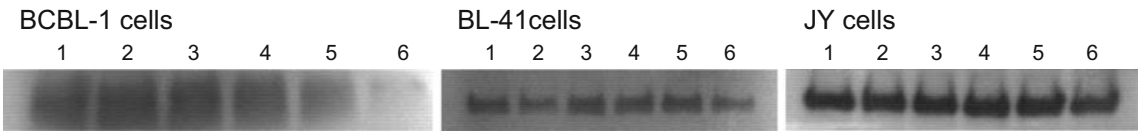

Fig. 2 Western blot analysis of BCL-2 protein expression in bcl-2 antisense oligonucleotide (*ASO*)-treated leukemia/lymphoma cell lines Cells were incubated with 05 and 1.0 μmol/l bcl-2 ASO (*lanes 2*, *5* and *3*, *6*, respectively) for 24 and 48 h (*lanes 1–3* and *4–6*, respectively). *Lanes 1* and *4* represent controls that were 1.0 μmol/l of scrambled oligonucleotide

Oligonucleotide Treatment of Suspension Cultures

For Western blot and DNA fragmentation studies, suspension cultures of JY, BCBL-1 and BL-41 cells were propagated for 24, 48, 64 and 72 h in the presence of either bcl-2 ASO or SCR-ON in a concentration range of 0.125 to 5.0 μmol/l. Untreated controls were also run.

Table 2 Changes in BCL-2 protein levels in oligonucleotide-treated cell lines and in primary ALL blasts

ASO treatment time/dose	Decrease in BCL-2 protein level relative to control (%)	Decrease in BCL-2 protein level relative to control (%)
JY cell line	bcl-2 ASO	SCR-ON
24 h/0.5 μM	0	0
24 h/1.0 μM	20	0
48 h/0.5 μM	25	0
48 h/1.0 μM	50	0
BL-41 cell line		
24 h/0.5 μM	0	0
24 h/1.0 μM	0	0
48 h/0.5 μM	10	0
48 h/1.0 μM	50	0
BCBL-1 cell line		
24 h/0.5 μM	0	0
24 h/1.0 μM	20	0
48 h/0.5 μM	40	0
48 h/1.0 μM	90	0
Primary leukemia cells		
Patient no. 1		
24 h/1.0 μmol	0	0
24 h/2.0 μmol	0	0
48 h/1.0 μmol	0	0
48 h/2.0 μmol	12	0
64 h/1.0 μmol	18	0
64 h/2.0 μmol	82	0
Patient no. 2		
24 h/1.0 μmol	0	0
24 h/2.0 μmol	0	0
48 h/1.0 μmol	0	0
48 h/2.0 μmol	0	0
64 h/1.0 μmol	0	0
64 h/2.0 μmol	25	0

ASO Antisense oligonucleotide; *SCR-ON* scrambled control oligonucleotide

Western Blot Analysis of The BCL-2 Protein

The 2×10^6 cells were treated with 30 μl ice cold lysis buffer [10 mM Tris (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM sodium ortho-vanadate], containing protease inhibitors [5 μg/ml Aprotinin, 1 μg/ml Pepstatin A, 2 μg/ml Leupeptin and 1 mM phenylmethysulphonyl-fluoride (PMSF)]. Protein extracts (5 mg/lane) were subjected to Western blot analysis according to standard methods by Bio Rad microassay (Bio-Rad, Richmond, CA, USA) [14]. Mouse monoclonal antibody to human BCL-2 was purchased from DAKO (Glostrup, Denmark) The bands were detected by ECL kit, according to the method of the manufacturer (ECL, Amersham-Pharmacia Budapest, Hungary).

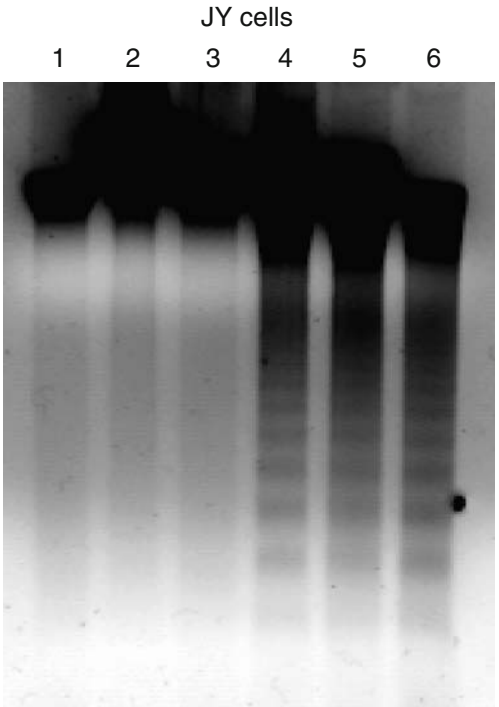

Fig. 3 Detection of genomic DNA-fragmentation during apoptosis (DNA-ladder) in bcl-2 antisense oligonucleotide (ASO)-treated JY cell line *Lanes 1* and *2* represent JY cells without antisense-oligonucleotide treatment at timepoint 0 and 48 h, *lane 3* represents JY cells treated with 2.0 μmol/l of bcl-2 ASO at timepoint 0. *Lanes 4–6* represent JY cells incubated with 0.5, 1.0 and 2.0 μmol/l of bcl-2 ASO for 48 h, respectively indicating well-marked DNA-fragmentation

DNA Fragmentation Assay

The 2×10^6 cells were collected by centrifugation (1,000 rpm, Jouan C/CR4-12, Horizontal Rotor, 10 min, 10°C) and washed twice with phosphate buffered saline [PBS (pH 7.4) Sigma, St. Louis, MO, USA]. DNA was extracted and subjected to agarose gel-electrophoresis by standard methods [15]. The gel was visualized under UV light and archived by AlphaImiger™ 2200.

Results

Effect of bcl-2 ASO on Colony Formation of Leukemia/Lymphoma Cell Lines

The spontaneous colony formation of the studied cell lines were inhibited by bcl-2 ASO in a dose-dependent manner. LC50 values of the three cell lines were within the same concentration range: 0.31 μmol/l for BL-41, 0.68 μmol/l for BCBL-1 and 0.86 μmol/L for JY. SCR-ON proved to be ineffective in inhibiting colony formation indicating the specificity of the bcl-2 ASO treatment (Fig. 1).

Changes in the BCL-2 Protein Level

Western blot analysis showed that bcl-2 ASO treatment, but not SCR-ON treatment resulted in a marked reduction in the BCL-2 protein expression in each three cell lines (Fig. 2, Table 2). The most prominent inhibition of BCL-2 expression was seen in BCBL-1 cells at a concentration of 1.0 μmol/l after 48 h of incubation. JY and BL-41 cells showed a moderate decrease in the level of BCL-2 at the same concentration. SCR-ON did not decrease the level of BCL-2 expression in the same concentration.

We have also subjected bcl-2 ASO-treated pimary lymphoblasts of two children with ALL to Western blot analysis. The bcl-2 ASO treatment caused a prominent decrease in the level of the BCL-2 protein at a low concentration (2 μmol/l) after 64 h (Table 2).

DNA Fragmentation

Characteristic apoptotic DNA fragmentation was observed in all of the three cell lines treated with ASO. Figure 3 shows a representative DNA fragmentation assay of untreated and ASO-treated JY cells. The SCR-ON controls did not induce DNA fragmentation.

Discussion

Several studies reported on a bcl-2 ASO-induced decrease in proliferative activity of cancer cells, including AML cell lines, renal cell carcinoma cells, laryngocarcinoma cells, non-small cell lung cancer cell lines and gastric cancer cells, accompanied by a decrease in their BCL-2 protein expression [16–18]. Interestingly, only few investigators assessed in vitro activity of bcl-2 ASOs in malignant cells of the B cell lineage. Jahrsdörfer et al. observed a significant inhibition of BCL-2 synthesis by bcl-2 ASO at 1 μmol/l in Daudi cells and primary B-CLL cells. ASO preparations, used by this group exhibited this activity only in the presence of cationic lipids [19]. Wang et al. used a higher concentration (20 μmol/l) of bcl-2 ASO induce apoptosis and downregulate BCL-2 protein expression in Raji cells [20]. However, bcl-2 ASO doses exceeding 20 μmol/l, may result in a non-specific inhibition of cell proliferation [21]. An effect of bcl-2 ASO on clonal proliferation of leukemia/lymphoma cell lines of the B cell lineage has not yet been reported.

Our data showed that bcl-2 ASO inhibited the colony formation of three leukemia/lymphoma cell lines of the B cell lineage in a dose-dependent manner. Maximal inhibition of clonal proliferation was achieved at a concentration of 0.75–5.0 μmol/l i.e. in a lower concentration than that was shown to exert non-specific inhibition of proliferation. The same concentration of bcl-2 ASO decreased also the expression of BCL-2 protein in each three cell lines, as demonstrated by Western blot. The inhibition of BCL-2 protein expression was observed not only in leukemia/lymphoma cell lines but, for the first time, also in primary lymphoblasts derived from pediatric ALL patients. There have no data been published about in vitro and in vivo use of bcl-2 ASO in childhood malignancies. Our results suggest that bcl-2 ASO alone, or in combination with cytostatic drugs and other biological response modifiers may represent a fruitful area for designing novel strategies against pediatric neoplastic diseases of the B cell lineage.

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