Survivin Antisense Oligodeoxy-Nucleotid Induces Apoptosis in Leukaemia Cell Line K562

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OBJECTIVE To investigate the effects of survivin antisense oligodeoxy-nucleotid (ASODN) on proliferation and apoptosis in the chronic myeloid leukemia cell line K562.

METHODS Different concentrations of an antisense oligodeoxy-nucleotid and control sequence (scrambled ODN) targeting the survivin gene were transferred into K562 by a lipofectin reagent. The MTT assay was used to measure the growth inhibitory rate, IC_{50}, and to observe the cytotoxicity of survivin ASODN in the K562 cells. The morphologic changes in the nucleus and the apoptotic rate were observed by Hoechst33342/PI staining. Caspase -3 activity was evaluated by a kinase activity assay. The changes of survivin protein expression after transfection were detected by Western blots.

RESULTS Eight hours after transfection, fluorescence in the K562 cells was well distributed. Treatment of the cells for 44 h with different concentrations of survivin ASODN produced a IC_{50} of 800 nmol/L. The growth inhibitory rate with 200, 400, 600 and 1000 nmol/L of survivin ASODN was 15.8±1.6%, 23.8±5.9%, 37.1±5.6% and 77.3±2.5% respectively. After 36 h of of survivin ASODN treatment, distinct morphologic changes characteristic of cell apoptosis such as karyopyknosis and conglomeration were observed by Hoechst33342/PI staining. Caspase -3 activity increased significantly after treatment of the cells with different concentrations of survivin ASODN ($P<0.01$)and following treatment with 800 nmol/L survivin ASODN, survivin expression decreased significantly.

CONCLUSION Survivin ASODN exerts an anti-cancer effect by inducing apoptosis in K562 leukaemia cells. Up-regulated expression of caspase-3 may play a role in this process.

KEYWORDS: surviving, antisense oligodeoxy-nucleotid, chronic myeloid leukaemia cell line K562, apoptosis, caspase.

Survivin is a member of a family of proteins that inhibit apoptosis and play critical roles in regulating the cell cycle and mitosis. Because of its high expression in essentially all human malignancies, and low or absent expression in most normal tissues, disrupting survivin function is a target for cancer gene therapy.[1-5] Development of safe and effective survivin antagonists for clinical use is hindered by a lack of understanding of the molecular mechanism by which survivin differentially affects apoptosis and cell division in normal and malignant cells. In this study we used the leukemia cell line K562, which overexpresses survivin, to examine the inhibitory effects of a survivin antisense oligodeoxy-nucleotid on survivin expression, cell proliferation, and apoptotic induction, with the prospect of appalying antisense oligodeoxy-nucleotids for tumor therapy.

MATERIALS AND METHODS

Cell culture

Chronic myeloid leukemia K562 cells were cultured in RPMI 1640 medium (Gibco Inc), supplemented with 10% calf serum, 100 U/L of penicillin and 80 U/L streptomycin, at 37℃ in a humidified atmosphere of 5% CO_2. The cells were regularly passaged to maintain exponential growth.

Antisense oligodeoxy-nucleotid preparation

An antisense oligodeoxy-nucleotid targeting the survivin gene and control sequences (scrambled ODN) was designed. The following antisense sequences were used: ASODN: 5'-TGG CTC CCA GCC TTC CAG CT-3' and scrambled ODN sequences were 5'-GGT CCT ACC CGC CTT CGA TC-3'. The oligodeoxy-nucleotid was 5' terminus FAM-conjugated and synthesized by Shenggong (Shanghai, China). The sequences were compared with sequences in the human EST(expressed sequence tag)database to confirm that no other genes were targeted. After the synthesis, ODNs were dissolved in PBS, and aliquots frozen at -20 ℃.

Transfection

The cells (1×10^5/ml) were plated in 6-well plates the day before cells were transfected. Survivin ASODN was transfected into the K562 cells mediated by a liposmal reagent. Transfection was accomplished using different concentrations of survivin ASODN per well along with the Tfx™-20 reagent (Promega), using methods according to the manufacturer's recommendations.

MTT assay

The K562 cells (1×10^4/ml)were plated in 96-well plates in RPMI 1640 containing 10% calf serum in a final volume of 0.1 ml. The next day the cells were treated with increasing concentrations of survivin A-SODN. After 44 h of culture, cell proliferation was assessed by the MTT assay. Following incubatious of the cells at 37℃ for 4 h, the reaction was stopped by the addition of 100 μl of DMSO and the absorbency was determined at 570 nm.

$$A_{570}=(A_{\text{control group}}-A_{\text{test group}})/A_{\text{control group}}\times100\%$$

Measurement of apoptosis by staining with Hoechst 33342/PI

The K562 cells(1×10^5/ml)were plated in 25 ml culture flasks. The next day the cells were treated with 200, 400, 600, 800 and 1000 nmol/L survivin ASODN. At 36 h following initiation of the culture, apoptosis was assessed by Hoechst33342/PI staining.

Caspase-3 activity assessment

After treatment with 600, 800 and 1000 nmol/L of survivin ASODN for 36 h, the K562 cells were centrifuged (450 *g*, 10 min at 4℃), washed twice with cold PBS, lysed in buffer, frozen and thawed 3 times, and centrifuged (15,000 *g*, 20 min at 4℃) to remove insoluble material. The supernatant was treated according to the CaspACE™ (Promega) assay system manufacturer's recommendations.

Western blots

After treatment with 800 nmol/L of survivin ASODN for 36 h, the cells were washed twice with cold PBS and lysed in assay buffer for 10 min. The extracts were centrifuged at 13,000 *g* at 4℃ for 15 min, and supernatants stored at -20℃ pending analysis. Protein extracts (50 μg) were heated (100℃, 3 min),separated by sulfacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred to nitrocellulose membranes (Sigma,USA) using a semi-dry transfer. The nitrocellulose membranes were blocked with Tris-HCl containing 0.05% Tween 20 at room temperature for 2 h, then incubated with anti-survivin antibodies (1:1000 dilution; Santa Cruz, CA, USA), and subsequently with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:500 dilution; RT, 1 h; Santa Cruz, CA, USA) and reacted for 2 h with specific antibodies in the same blocking solution. The membranes were reacted with survivin antibody. Finally the membranes were developed with DAB and incubated until color developed sufficiently.

Statistical analysis

All statistical analyses were performed using a one-way ANOVA test. Differences between experimental groups were determined by the Tukey's test, with values of $P<0.05$ considered to be significant.

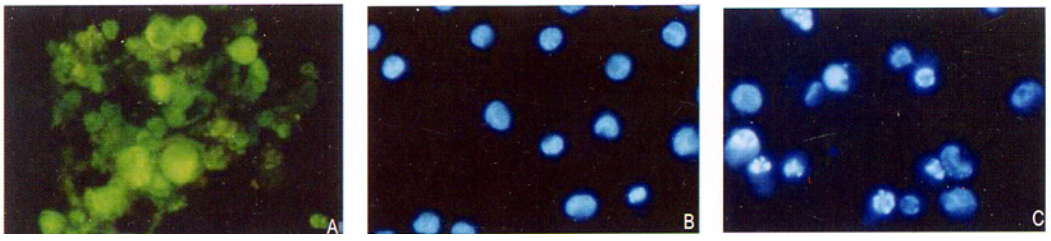
RESULTS

Transfection

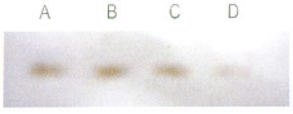
After 8 h of transfection, the fluorescence in the K562 cells was symmetrical (Fig.1A), indicating that the transfection was successful.

Anti -proliferative effect of survivin ASODN on K562 cells

When the K562 cells were treated with 200, 400, 600,



A: Survivin ASODN was transferred into K562 Cells B: Untreated K562 cells C: K562 cells were treated with 800 nmol/L survivin ODN for 36 h
Fig.1. K562 cells × 200.



A: Control group B: Scrambled ODN control group
C: Lipofectin control group D: Survivin ASODN group(800 nmol/L)
Fig.2. Expression of survivin in K562 cells examined by Western blots.

800 and 1,000 nmol/L survivin ASODN for 44 h, the inhibitory rates increased with each level of ASODN (Table1). There were significant differences among all values(P<0.01). Survivin ASODN inhibited the growth and proliferation of the K562 cells in a concentration-dependent manner.

Table 1. Growth inhibitory rates of K562 cells treated with survivin ASODN ($\bar{x}\pm s$)

Groups (concentration nmol/L)	A_{570} (n=8)	Inhibitory rates (%)
Blank control group	0.232 ± 0.024	-
Control group	1.150 ± 0.233	-
Lipofectin control group	1.164 ± 0.124	-
Scrambled ODN group 800	1.102 ± 0.093	-
Survivin ASODN group 200	0.974 ± 0.032	15.80 ± 1.55
Survivin ASODN group 400	0.815 ± 0.061*	23.78 ± 5.87
Survivin ASODN group 600	0.73 ± 0.0240*	37.09 ± 5.58
Survivin ASODN group 800	0.577 ± 0.024*	50.64 ± 8.60
Survivin ASODN group 1000	0.274 ± 0.015*	77.26 ± 2.51

* P<0.01 *vs.* control group

Apoptotic induction in K562 cells

After 36 h of treatment with different concentrations of survivin ASODN, distinct morphologic changes indicative of apoptosis such as karyopyknosis and conglomeration were observed by Hoechst33342/PI staining (Figs.1B,1C). The apoptotic rates following treatment with 600, 800 and 1000 nmol/L survivin ASODN were (36.6±1.1%), (57.5±1.8%) and (65.6±1.5)%, respectively.

The activity of Caspase-3

Compared with control group the activity of Caspase-3 was increased significantly (Table 2) after the K562 cells were treated with different concentrations of survivin ASODN (P<0.01).

Table 2. Effect of survivin ASODN on caspase-3 activity of K562 cells ($\bar{x}\pm s$)

Groups (concentration nmol/L)	A_{570} (n=8)	△A
Control group	0.055 ± 0.003	-
Scrambled ODN group 800	0.062 ± 0.003	-
Survivin ASODN group 600	0.180 ± 0.017	0.111 ± 0.019*
Survivin ASODN group 800	0.770 ± 0.047	0.693 ± 0.049*
Survivin ASODN group 1000	0.480 ± 0.023	0.403 ± 0.022*

* P<0.01 *vs.* control group

Down-regulation of survivin expression

Survivin ASODN-treated cells were collected and Western blots performed to assess the expression of survivin. We found that survivin ASODN significantly inhibited the expression of the survivin protein (Fig.2).

DISCUSSION

In the present study we have demonstrated that reduction of survivin expression in K562 leukemia cells is a potential target for tumor therapy. The human survivin gene spans 14.7 kb on the telomeric position of chromosome 17. The gene is localised on band q25 which is essentially comprised of an NH2 terminal BIR domain typical of anti-apoptotic proteins, with a C-termi-

nal amphipathic alpha helical coil. Studies have confirmed the high incidence of survivin expression in acute myeloid leukemia (AML)and found a very high survivin expression in the majority of patients with chronic myeloid leukemia.[6,7] Its expression is also abnormal in the myelodysplastic syndrome (MDS). Survivin may play a role in promoting excessive cell viability and contribute to the altered homeostatic balance between cell growth and cell death.[8] Newly diagnosed patients with childhood AL had a higher expression of survivin which may become a maker for the treatment and evaluation of the childhood AL patients.[9]

The absence of survivin from most healthy tissues makes it a target for tumor therapy. Several preclinical studies have demonstrated that down-regulation of survivin expression/function, accomplished through the use of antisense oligonucleotides, small interfering RNA and cyclin-dependent kinase inhibitors, increased the apoptotic rate, reduced tumor-growth potential and sensitized tumor cells to chemotherapeutic drugs. These findings were based on different action mechanisms in models of various human tumor types.[5] ASODN represents a useful experimental approach for manipulating gene expression, and recently some antisense compounds have shown anticancer efficacy in numerous preclinical studies. It has been reported that suppression of survivin expression using antisense oligonucleotide induces tumor cell apoptosis. Further analysis of the importance of survivin antisense oligonucleotide localization is needed in order to define better the therapy target. Lu et al.[10] showed that inhibition of survivin expression accomplished through the use of antisense oligonucleotides was able to increase the sensitivity of H460 lung cancer cells to ionizing radiation. More recently, it was demonstrated that antisense ODN targeting the survivin gene could significantly inhibit the proliferation of MG-63 cells and increase cell apoptosis.[4] Chen et al.[11]found that transfection of ASODN targeted to the promoter region of survivin mRNA can down-regulate the expression of the survivin protein and mRNA significantly in SMMC-7721 cells and inhibit their proliferation .

The cell cycle-dependent expression of the survivin gene in mitosis suggests a role for survivin in promoting cell proliferation; however, recent data point to a more selective role of survivin in antagonizing mitochondria-dependent apoptosis,[12] and a mitochondrial pool of survivin has recently been shown in cancer cells.[13] In our study, we chose survivin as the molecular target and designed ASODN to observe its influence on growth and apoptosis of the chronic myeloid leukemia cell line K562. Our results showed that survivin ASODN (600~1,000 nmol/L) inhibited the expression of survivin and K562 cell growth effectively, and induced cell apoptosis significantly. We propose that survivin ASODN inhibits K562 cell growth by inducing apoptosis.

Survivin is selectively expressed in various malignant tumors, but its role in carcinogenesis and the mechanism by which it may function in a tumor is not clear. Apoptosis is mediated by caspases, a family of cysteine proteases that become activated by proteolysis and cleave multiple cellular substrates. Survivin could act directly on caspases and mainly suppress the activity of Caspase-3 and Caspase-7,[14] but not of the upstream initiator protease Caspase-8. Survivin can thereby down-regulate, directly or indirectly, both death-receptor-mediated and mitochondria-mediated pathways of apoptosis.[15] We suggest that up-regulation of the expression of Caspase-3 might be one of the possible mechanisms contributing to survivin ASODN-induced apoptosis.

Our data indicate that survivin may be an important gene therapeutic target in tumors including chronic myeloid leukaemia, and that survivin ASODN might become a useful therapeutic agent in the future.

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