Studies of Liposomal bcl–2 Antisense Oligode–oxynucleotide Induction of Apoptosis in Raji Cells

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OBJECTIVE To explore the effect of liposomal G3139 and transfected antisense phosphorothioate oligodeoxynucleotides directed against the coding region of the bcl–2 messenger RNA and the translation site on apoptosis in Raji cells.

METHODS Cytotoxic effects were measured by use of the MTT method; the expression levels of Bcl–2 protein were assayed by immunofluorescence using a fluoresce isothiocyanate label. Apoptosis was determined by morphological observation and flow cytometric analysis.

RESULTS The 2 antisense oligonucleotides and G3139 can reduce Bcl–2 protein levels and Raji cell viability (IC50=4.54, 4.72 and 4.26 μmol/L, respectively), and induce apoptosis. A scrambled sequence control oligonucleotide and empty liposomes did not alter cell viability, Bcl–2 protein expression or apoptosis rates. There was no difference in reducing Bcl–2 protein levels and apoptosis rates found among the 3 antisense oligonucleotides.

CONCLUSION The 2 antisense oligodeoxynucleotides of bcl–2 messenger RNA can effectively induce apoptosis of Raji cells. The 2 antisense sequences and G3139 have a similarity in their antisense effect.

KEYWORDS: bcl–2, antisense oligonucleotide, G3139, Raji cells, apoptosis.

Deregulated expression of Bcl-2 prevents apoptosis and thus contributes to the majority of human cancer development. Expression of Bcl-2 has been observed in the majority of human cancer specimens and cell lines and has been associated with drug resistance.[1,2] It has been shown that antisense-mediated reduction in Bcl-2 protein levels could ultimately induce a lower apoptotic threshold and restore chemosensitivity in a variety of hematologic malignancies, including acute leukemia and non-Hodgkin lymphoma.[3,4] The bcl-2 antisense oligonucleotides (ASODN), G3139 which have been developed by Genta Inc. are currently in phase III clinical trials. In our lab, 2 novel antisense sequences have been identified in the translation initiation and the coding region of bcl-2 mRNA that most effectively down-regulate expression of the bcl-2 proto-oncogene.[5] The study was designed to compare further the effect of the 2 novel ASODN of bcl-2 mRNA and G3139 on apoptosis in Raji cells by liposomal transfection. Our findings may be useful in searching for a new bcl-2 antisense drug.
MATERIALS AND METHODS

Cell line
The Raji cell line was kindly provided by Dr. Zhu Xiaofeng, Sun Yat-Sen University Cancer Center. The cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37℃ under 5% CO2 in a humidified incubator.

Synthesis of oligonucleotides
The sequence of the ASODN targeting the coding region of the bcl-2 mRNA was 5'-ATCCTCCCGCAT-CCAGTTCACC-3' (ASODN1). The sequence of the ASODN targeting the translation initiation region of the bcl-2 mRNA was 5'-AGCGTCCGCCATCCTTCC-3' (ASODN2). For a control, nonsense ODN (NSODN) were used in the study. The sequence was 5'-CACCCCAATTCTTCCGCC-Y. G3139 was used for a positive control. The sequence of G3139 is 5'-TCTCCCAGCGTGCGCCAT-3'. Phosphorothioate oligonucleotides (ODN) were synthesized and purified by Shanghai Sangon Biology Engineer Corporation. Oligofectamine was purchased from Invitrogen.

MTT assay
To determine cell viability, Raji cells were seeded at a density of 1 × 10⁴ cells per well in 96-well plates. Six hours later, liposomal bcl-2 ASODN1, ASODN2, NSODN and G3139, or empty liposomes were added to the cells at a final concentration of 0.8 to 12.8 μmol/L. After 4 days of culture, 100 μg of MTT solution was added to each well in the culture plate. After 4 h, cells were centrifuged (2000 × g for 5 min) and resuspended in a final volume of 200 μl of DMSO. Cell viability was measured with a spectrophotometer at an absorbance of 490 nm.

Quantitation of Bcl-2 protein by flow cytometry
Raji cells were collected by centrifugation (2000 × g for 5 min). Cells were washed twice and fixed in 4% formaldehyde (Sigma) for 30 min on ice. Cells were then washed in cold phosphate-buffered saline (PBS) before being added to 10 μl of fluorescein isothiocyanate (FITC)-conjugated anti-Bcl-2 (DAKO, Carpinteria, CA) for 30 min at room temperature in the dark. After washing twice with PBS, the cells were separated by flow cytometry (Becton Dickinson) and the percentage of cells with positive staining for the Bcl-2 protein was determined.

Examination of cell morphology
Raji cells were harvested, and fixed in methanol. Morphology was studied with Gimesa stain. The morphology of cells was examined under a light microscope.

Detection of apoptotic cells by flow cytometry
The Raji cells were collected by centrifugation (2000 × g for 5 min), washed with PBS and resuspended in a final volume of 200 μl of ice-cold PBS. The cells were resuspended in 1 ml of 70% ethanol in PBS with vigorous mixing. Fixed cells were incubated in the dark at 4℃ overnight. The cells were rehydrated in 500 μl of PBS for 25 min and then stained for 5 min with propidium iodide before flow analysis. The cells were assessed with respect to their red-fluorescence profile (575 nm) using 488 nm excitation at 150 mW from a Coherent Enterprise Laser of a FACS vantage instrument (BD). DNA histograms were produced using LYSIS II software.

Statistical analysis
Results were expressed as x ± SD and analyzed using ANOVA, using a significance level of P<0.05.

RESULTS

Cytotoxic effects of bcl-2 ASODN on the Raji cells
The viability of Raji cells was effectively reduced by bcl-2 ASODN1, ASODN2, and G3139 in a dose-dependent fashion. NSODN and empty liposomes did not affect the cell viability. The IC₅₀ of the bcl-2 ASODN1, ASODN2, and G3139 was (4.54 ± 0.18) μmol/L, (4.72 ± 0.18) μmol/L and (4.26 ± 0.13) μmol/L, respectively. There was no difference in the IC₅₀ among bcl-2 ASODN1, ASODN2 and G3139.