

Bcl-X_L Antisense Sensitizes Human Colon Cancer Cell Line to 5-Fluorouracil

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Resistance to 5-fluorouracil (5-FU) has been frequently found in the treatment of digestive tract cancer patients. Our previous study suggested that high expression of endogenous Bcl-X_L, might be associated with resistance to 5-FU in colorectal cancer. The aim of this study is to analyze the role of Bcl-X_L in 5-FU resistance and to explore a new therapeutic strategy using Bcl-X_L antisense. First, western blot analysis shows that Bcl-X_L rather than Bcl-2 is overexpressed in primary adenocarcinoma of colon. Second, when Colo320 cells, with undetectable endogenous Bcl-X_L expression, were transfected with *Bcl-X_L* gene, they acquired high resistance to 5-FU. Finally, antisense oligodeoxynucleotides (ODNs) that targeted the start codon of Bcl-X_L mRNA (AS1) prove to be the most effective in DLD1 cells with high endogenous Bcl-X_L expression. Bcl-X_L protein expression was decreased in a dose-dependent manner when the cells were treated with AS1 ODNs, while non-sense and sense controls and 5-FU had no effect on Bcl-X_L protein. 5-FU treatment induced a level of apoptosis 10-fold higher in DLD1 cells than in untreated control cells, while the same dose of 5-FU induced a 55-fold higher level of apoptosis in DLD1 cells treated with Bcl-X_L antisense oligodeoxynucleotides ($P=0.0003$). Moreover, AS1 ODNs coupled with 5-FU decreased viable colon cancer cells 40% more than did 5-FU alone ($P<0.05$). These results suggest that Bcl-X_L is an important factor for 5-FU resistance and the suppression of Bcl-X_L expression by the specific antisense ODNs can increase the sensitivity of colon cancer cells to 5-FU.

Key words: Bcl-X_L — 5-Fluorouracil — Antisense — Apoptosis — Resistance

Digestive tract tumors, like other solid tumors, respond poorly to chemotherapy. 5-Fluorouracil (5-FU), which is considered the standard anti-cancer agent in colon cancer, has less than 20% rate of complete response in metastatic colon cancer. Resistance to apoptosis induction is now accepted as one of the mechanisms responsible for resistance to anti-cancer drugs,¹⁾ and the Bcl-2 family proteins are critical regulators of apoptosis.²⁾ Bcl-2 and Bcl-X_L can block apoptosis by inhibiting molecules required for the activation of apoptosis effectors. Both proteins have been associated with increased resistance to anticancer agents. For example, Bcl-X_L overexpression in neuroblastoma cells³⁾ can protect these cells from apoptosis induced by various chemotherapeutic compounds. Similarly, Bcl-2 overexpression can inhibit cell death induced by chemotherapy.⁴⁾ Experiments using antisense technology, by reducing endogenous Bcl-2 levels, have confirmed the results of the overexpression experiments by showing an inverse correlation between chemosensitivity and Bcl-2 levels.⁵⁾ Together, these results suggest that modulation of the expression of the Bcl-2 family might be effective in overcoming the resistance to anti-cancer drugs.

Antisense oligodeoxynucleotides (ODNs) are short, single-stranded DNA molecules that can reduce gene expression by forming RNA-DNA duplexes, thereby averting mRNA translation. Antisense ODNs targeted to activated oncogenes might also have a therapeutic role in the treatment of human malignancies. After early criticisms, some consensus has been achieved in this research field, including a need to screening multiple ODNs designed to hybridize to different regions on the target mRNA, appropriate number of controls for the antisense ODNs, and dose-response curves evaluating the inhibition of target proteins.^{6,7)} Antisense ODNs now have been used with some therapeutic success in animal models of cardiovascular⁸⁾ and neoplastic disorders.^{5,9)} More recently, phase I clinical trials have been reported in some types of human cancers.⁹⁾

In this study we first analyzed the Bcl-2 and Bcl-X_L protein expression pattern in adenocarcinoma of the colon. Next, we studied the effect of *Bcl-X_L* gene transfection in a cell line with undetectable endogenous Bcl-X_L. Finally, we aimed to determine whether the chemosensitivity of human colon cancer cells to 5-FU could be increased by decreasing Bcl-X_L expression with antisense therapy.

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MATERIALS AND METHODS

Patients and samples The surgically resected specimens were obtained from unselected patients for whom frozen tumor specimens were available and with a diagnosis of colorectal cancer, seen at the Department of Surgical Oncology, University of Tokyo Hospital during the period of 1995 to 1997. For western blot studies, fresh surgical tumors were cut for routine histology, and one further portion was carefully selected from the most viable part of the tumor, along with adjacent normal colonic mucosa, to be immediately frozen in liquid nitrogen, transferred and stored at -80°C until analysis. Patients had received neither chemotherapy nor radiation therapy before surgery.

Reagents and antibodies 5-FU was provided by Kyowa Hakko Kogyo Co., Ltd., Tokyo. Mouse anti-FLAG M2 mouse monoclonal antibody (mAb) and anti- β -actin mAb were purchased from Sigma (Saint Louis, MO). Anti-Bcl-2 (clone 7), and anti-Bcl- X_L mAb were from Transduction Laboratories (Lexington, KY), and anti-Bax mAb (clone 4F11) from MBL Hiteclone (Nagoya).

Cell lines and vector transfection Colo320 and DLD1, human colon cancer cell lines, were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) in a humidified incubator at 37°C with 5% CO_2 . The control expression vector pEF FLAG pGK puro, and vector containing human Bcl- X_L with the FLAG tag (both generous gifts from D. Huang and A. Strasser) have been described before.¹⁰⁾ Colo320 cell line was transfected with these vectors by using the non-liposomal lipid Effectene (Quiagen, Hilden, Germany), and stable transfectants were selected in medium containing puromycin (4 $\mu\text{g}/\text{ml}$). The cell line was single-cell-cloned using limiting dilution culture, and western blotting was used to identify clones expressing high levels of Bcl- X_L .

Antisense ODNs High-performance liquid chromatography (HPLC)-purified antisense and control ODNs, all of them with phosphorothioate modification, were purchased from Sawady Technology (Tokyo). Six antisense ODNs directed to the sequences in Bcl- X_L (GenBank accession number: Z231115) were evaluated: AS1 (antisense ODN with phosphorothioate modification 1, targeting Bcl- X_L mRNA): GTT GCT CTG AGA CAT TTT; AS2: GGC ACT GGG GGT CTC CAT CT; AS3: TGT ATC CTT TCT GGG AAA GC; AS4: CTT TCC ACG CAC AGT GCC; AS5: TCA TTT CCG ACT GAA GAG TG; AS6: CGG AGG ATG TGG TGG AGC AG. The controls were NS1 (nonsense 1 control): AAG TCG AGC CTC CTC GTT and SE1 (sense 1 control for AS1): AAA ATG TCT CAG AGC AAC. A BLASTN search of a database containing all sequences in the GenBank and European Molecular Biology Laboratory (EMBL) database revealed no sequence homology of those ODNs to other human genes.

Treatment of cells with antisense ODNs The ODNs were either directly added to the dishes (naked) or ODNs were delivered to DLD1 cells in the form of complexes with Effectene according to the manufacturer's specifications with the following modifications. In preliminary experiments, different concentrations of ODNs either naked or complexed with Effectene were tested to optimize the uptake of ODNs by the cells. In brief, 1 μM ODNs was mixed with 8 μl of Enhancer, and allowed to form a complex for 5 min. Then, 10 μl of Effectene was mixed with the ODNs-Enhancer mixture; this ratio of ODNs-Enhancer 1:10 Effectene was held constant in all experiments. Antisense controls ODNs were treated with the same protocol. Cells were incubated at 37°C for different periods of time, depending on the experiments. The accumulation of FITC-labeled ODNs (Sawady Technology) in DLD1 cells was monitored using a fluorescence microscope. At 6 h, AS1-FITC ODNs transfected into the DLD1 cells with Effectene were seen as fine speckles or large aggregates of ODNs within the cytoplasm of most of the cells. In clear contrast, naked AS1-FITC ODNs had accumulated in a very few cells and in lower amounts than those ODNs complexed with lipids. AS1-FITC ODNs transfected with Effectene were still present inside the cells after 72 h of continuous incubation at 37°C with RPMI 1640 containing 10% FCS. We concluded that ODNs should be transfected complexed with Effectene, and a single dose of ODNs would be appropriate for experiments requiring 72 h of continuous incubation (data not shown). The optimal dose of AS1 was determined on the basis of the cytotoxicity of ODNs mixed with Effectene. Doses as high as 10 μM were extremely toxic to the cells with virtually no cell left alive. On the other hand, AS1 at the dose of 1 μM had a much lower cytotoxicity as compared with 10 μM ; and, at the same time, it was more toxic than the NS1 control ODNs (unpaired *t* test, $P < 0.05$). NS1 at 1 μM exhibited similar toxicity to the lipids alone. Because of this pattern of toxicity we chose to perform the experiments with 1 μM of AS1 and its control ODNs.

Western blotting analysis Protein was extracted from the frozen tumor under stringent conditions to avoid degradation and contamination. A set consisting of equal amounts of protein (10 μg) from each cell lysate was analyzed by immunoblotting as previously described.¹¹⁾ Digitized western blot images were analyzed with Luminous Imager software (Aisin Cosmos, Tokyo). The protein content was expressed using the densitometry score (arbitrary units). Following incubation of colon cancer cell lines in either the absence or the presence of antisense ODNs and/or 5-FU for a given period of time, total cell lysates were harvested and equivalent amounts of proteins were used for western blotting. The relative expression was calculated after correction for the background and the amount of pro-

tein loaded by means of normalization against β -actin. Relative expression is the ratio of ODN/5-FU treated cells to untreated control cells. Values are representative of at least two independent experiments.

Apoptosis analysis The cells were cultured in either the absence or the presence of 5-FU at the IC₅₀ (21 μ M) and/or ODNs (1 μ M) for two or three consecutive days. Apoptosis was evaluated by three different methods. First, apoptosis was identified by flow cytometric analysis of annexin V-Fluos (Boehringer Mannheim, Mannheim, Germany). In brief, about 10⁶ cells were labeled with a mixture of annexin V and propidium iodide in incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂ buffer). The cells were then analyzed with a flow cytometer (Becton Dickinson, Sunnyvale, CA). The results shown are the levels of live cells compared with cells undergoing apoptosis (staining for annexin) after excluding clusters of necrotic cells (staining for propidium iodide). Further, apoptosis was confirmed by morphological analysis by staining the cells with acridine orange (5 μ g/ml, Sigma) as described elsewhere¹²⁾ and observed by fluorescence microscopy. Finally, apoptosis was quantified by a Cell Death Detection ELISA Kit (Boehringer Mannheim) according to the manufacturer's specifications. Apoptosis level is the ratio of test (AS ODNs and/or 5-FU) to untreated control cells and is represented as fold difference in apoptosis level after setting the level of DLD1 control cells to 1.0.

Growth-inhibition assays Cytotoxicity was determined by the MTS assay (Promega, Madison, WI). The viable cell number was determined by trypan blue exclusion test. In brief, DLD1 was seeded in 6-well plates at densities of 10⁵ cells per well. After 24 h, ODNs were transfected into the cells with Effectene using the protocol described above. At the same time, 5-FU at the IC₅₀ dose was added to the wells. The NS1 and SE1 ODNs (1 μ M), and control for the transfectant agent (Effectene) were used in control experiments.

Statistical analysis Statistical analysis was performed with Stata 6.0 (Stata, College Station, TX). Paired or unpaired *t* tests were used to assess the statistical significance of differences in continuous variables. Two-sided test was used and the α level was set at 0.05.

RESULTS

Differential Bcl-2 and Bcl-X_L protein expression in primary adenocarcinoma of colon A total of 24 colonic tumor samples and corresponding normal mucosa were analyzed in this study. Bcl-X_L protein expression was detected at the expected 26 kDa in most cancer samples, and it was not expressed in most normal mucosa (Fig. 1A); this difference was statistically significant (Fig. 1B). Conversely, Bcl-2, which was detected as a single or

doublet band at 26 to 30 kDa, was found in most normal mucosa samples, but in only a few cancer samples (Fig. 1A). After normalization of protein expression with respect to β -actin, it can be seen that there was a clear and significant decrease in Bcl-2 protein expression from normal mucosa to cancer samples (Fig. 1B). The additional band seen at 30 kDa might correspond to a phosphorylated form of Bcl-2 protein.¹³⁾

Overexpression of Bcl-X_L in Colo320 cells with undetectable endogenous Bcl-X_L Our previous study¹¹⁾ showed that Colo320 expressed undetectable Bcl-X_L protein and had high sensitivity to 5-FU. Therefore, this colon cancer cell line was selected for the transfection study using *Bcl-X_L* gene. After the transfection and generation of stable transfectants, clones with high expression levels of

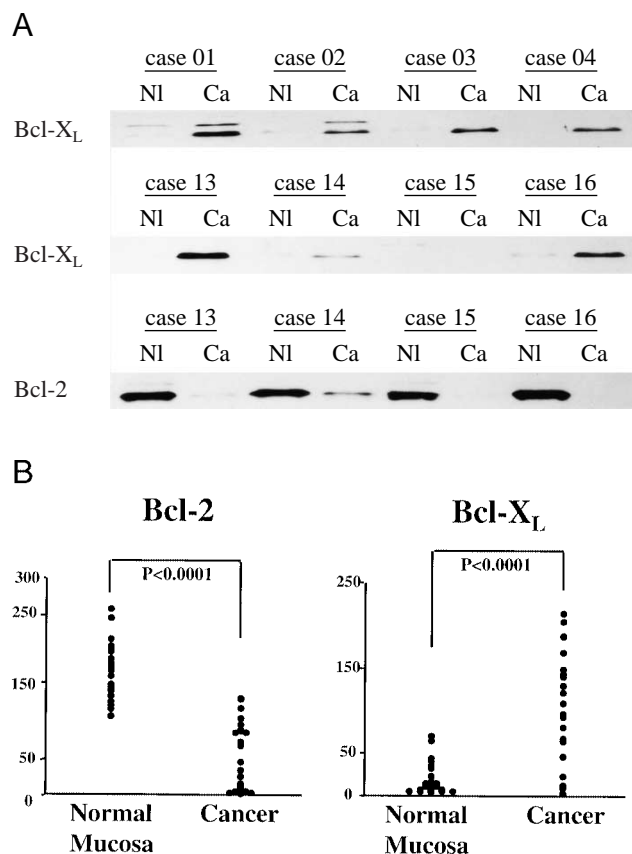


Fig. 1. (A) Expression of Bcl-X_L (upper and middle panels) and Bcl-2 (lower panel) proteins in colorectal cancer and its adjacent normal mucosa. Equal amounts of proteins were applied in each lane and analyzed by western blot. (B) Expression of Bcl-X_L and Bcl-2 proteins in colorectal cancer and its adjacent normal mucosa. Densitometric scanning was performed on the series of blots shown in Fig. 1A. The expression levels were calculated after correction for the background and are expressed in arbitrary units. The *P* values were calculated by paired *t* test; *P* < 0.0001 for both Bcl-X_L and Bcl-2 proteins.

Bcl-X_L (Fig. 2) were selected and the correlation between Bcl-X_L expression and 5-FU resistance was examined using cytotoxicity assay. When cells were exposed to 5-FU for 72 h, the IC₅₀ of vector-transfected control cells was 1.1 μM, while cells overexpressing Bcl-X_L (Fig. 2, lane 2) showed an IC₅₀ of 5.1 μM (unpaired *t* test, *P*=0.001). A similar result was observed with a different clone (Fig. 2, lane 3). Thus, this result shows that Bcl-X_L is able to increase the resistance of Colo320 to 5-FU.

Development and characterization of antisense ODNs to Bcl-X_L We previously¹¹⁾ demonstrated that DLD1 human colon cancer cell line expressed high levels of endogenous Bcl-X_L protein and was resistant to 5-FU treatment. The six different antisense ODNs were designed to hybridize to the start codon, coding region and 3' untranslated region of the human Bcl-X_L mRNA. Their ability to inhibit Bcl-X_L expression was tested by western blot. Fig. 3 shows that AS1 was the most effective to suppress the Bcl-X_L protein expression in DLD1 cells and it

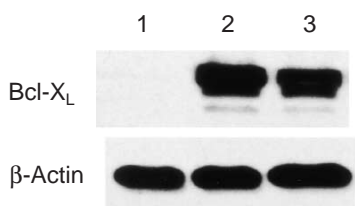


Fig. 2. Western blot analysis of Colo320 cells transfected with either empty vector (1) or the vector with a Bcl-X_L cDNA insert (2 and 3). Twenty micrograms of total cellular protein was used for western blot analysis.

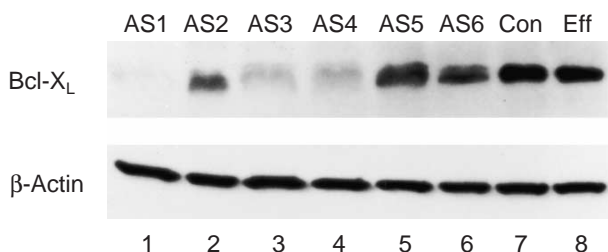


Fig. 3. Immunoblotting of Bcl-X_L protein after ODNs treatment. DLD1 cells were treated with 1 μM of multiple ODNs (lanes 1–6), targeting different regions of Bcl-X_L mRNA, for 48 h, and equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and blotted with an anti-Bcl-X_L (upper panel) or β-actin (lower panel) mAb. β-Actin was used as a loading control for the western blot. Also shown, con, control unexposed cells (lane 7); and, Eff, Effectene alone as a control for the transfectant agent (lane 8).

was selected for further analysis. DLD1 cells were treated with AS1 ODN and Bcl-X_L protein levels were analyzed to assess further the effectiveness and specificity of the antisense phosphorothioate ODNs. Fig. 4A shows a dose-response analysis, from which it is clear that Bcl-X_L antisense ODNs decreased the Bcl-X_L protein level in a concentration-dependent manner. In contrast, neither the transfecting agent (Effectene) alone nor the NS1 and SE1 control ODNs had any relevant effect on the levels of Bcl-X_L protein. Densitometric quantification analysis of the protein levels after normalization to the β-actin levels and comparison with untreated control cells showed that AS1, at the dose of 1 μM, caused about a 64% reduction in Bcl-X_L protein levels. Moreover, the same membrane used to detect Bcl-X_L protein was stripped of bound antibody and

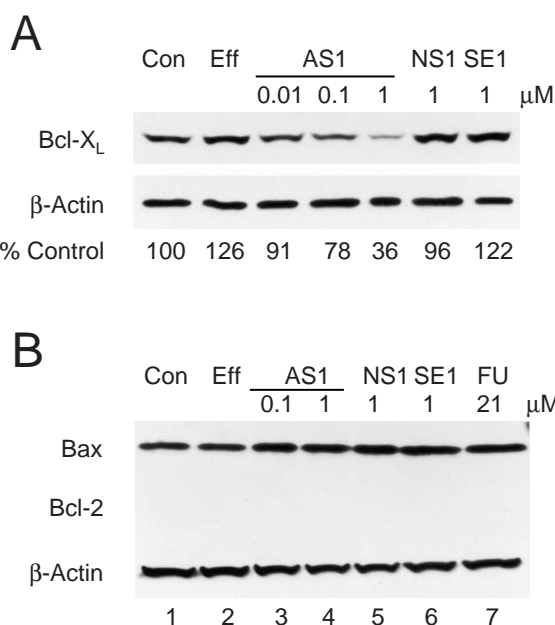


Fig. 4. (A) Dose-response analysis of Bcl-X_L protein after treatment with increasing doses of AS1 ODNs. DLD1 cells were treated with indicated concentrations of ODNs for 48 h, and equal amounts of extracted proteins were analyzed for Bcl-X_L (upper panel) or β-actin (middle panel). β-Actin was used as a loading control for the western blot. The relative expression is given as % of treated cells to untreated control cells (lower panel). Also shown, con, control unexposed cells; and Eff, Effectene alone for control for the transfectant agent. (B) Effects of AS1 and its control on Bcl-2 and Bax protein levels. Western blot analysis of equal amounts of cell lysates from DLD1 after 48 h of exposure to the indicated doses of AS1 ODNs (lanes 3 and 4), nonsense control (lane 5), sense control (lane 6) and 5-FU (lane 7) was carried out with anti-Bax (upper panel) or Bcl-2 (middle panel) mAb.

re-probed for β-actin protein. Neither Bcl-X_L AS1 nor its controls caused any alteration of β-actin levels, suggesting selectivity for the targeted protein. In a further assessment of the specificity of this down-regulation we checked the protein levels of Bcl-2 and Bax, two proteins of the same family of Bcl-X_L. Fig. 4B shows that there were no significant alterations of the levels of those proteins. Treatment with 5-FU had also no effect on Bcl-X_L protein (data not shown). Together, these results confirm the selectivity of the AS1 ODNs in reducing the expression of Bcl-X_L protein in DLD1 colon cancer cell line.

Decreased expression of Bcl-X_L increased 5-FU-induced apoptosis Overexpression of Bcl-X_L protein protects cells from undergoing apoptosis induced by several

agents, including chemotherapeutic drugs.^{3,14)} Consequently, it is expected that down-regulation of Bcl-X_L is associated with an increased rate of apoptosis induced by anticancer agents. Annexin V-Fluos analysis of the early phase of apoptosis by flow cytometry (Fig. 5A) showed that DLD1 cells treated with AS1 ODN were induced to undergo apoptosis, and the rate of apoptosis was increased when the cells were exposed concomitantly to AS1 ODN and 5-FU. Because morphological criteria are considered the gold standard in the evaluation of apoptosis, we assessed the morphology of cells exposed to the ODNs alone or combined with 5-FU by acridine orange staining of the cells. Cells with apoptotic characteristics¹⁵⁾ were found among cells exposed to AS1 (Fig. 5B) and to AS1

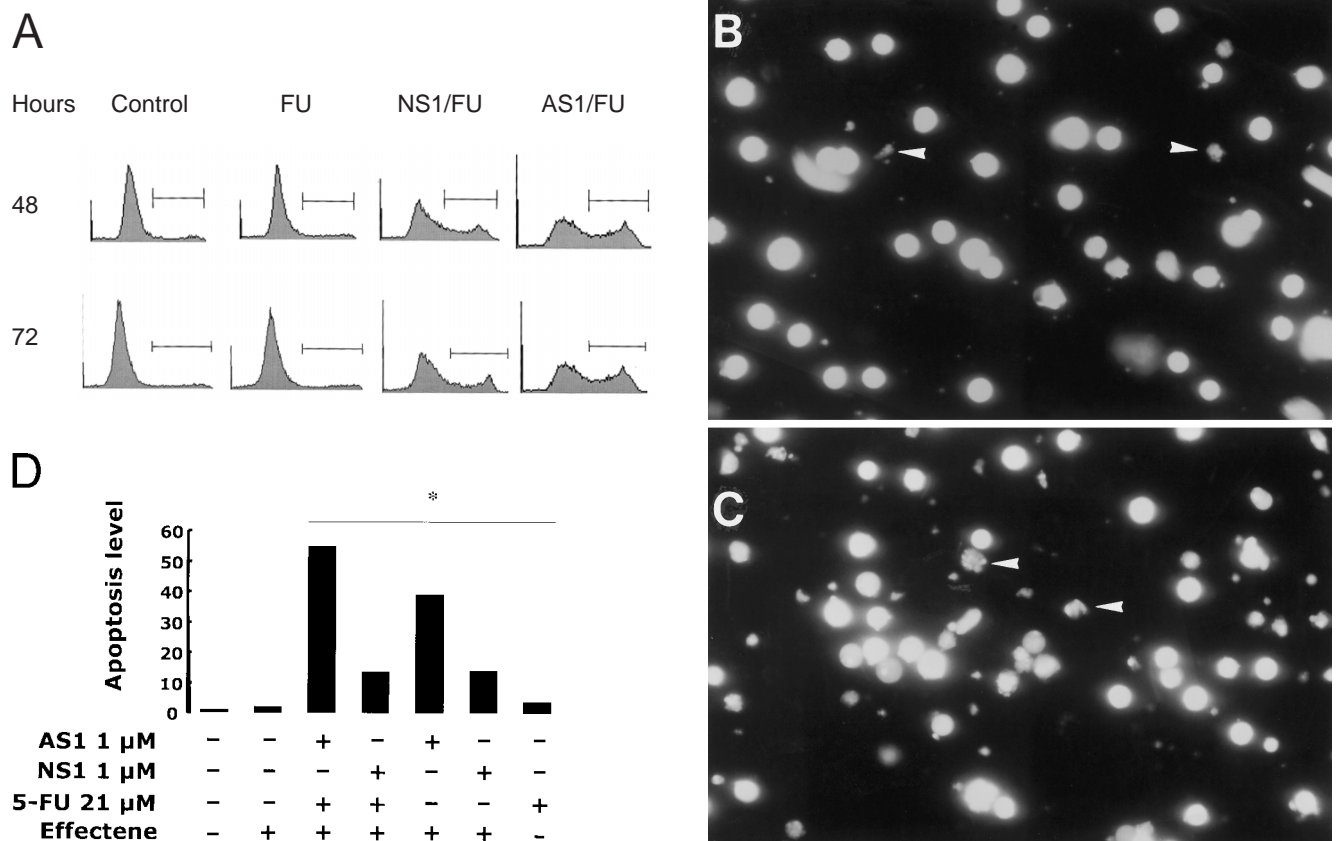


Fig. 5. Effects of AS1 on 5-FU-induced apoptosis. (A) DLD1 cells were incubated with 21 μM 5-FU and with 5-FU and 1 μM AS1 or 1 μM NS1 control, and after 48 and 72 h of treatment, 10⁶ cells labeled with annexin V-Fluos were analyzed by FACScan flow cytometry to detect early apoptotic cells. For acridine orange analysis of cell morphology, DLD1 cells were exposed to (B) 1 μM AS1 ODNs; or (C) 1 μM AS1 ODNs and 21 μM 5-FU for 72 h, and unfixed cells stained with 5 μg/ml of acridine orange were examined by fluorescence microscopy. (D) Quantification of apoptosis induced by Bcl-X_L antisense in DLD1 cells. Cells were treated with 1 μM AS1, 1 μM NS1 control or 21 μM 5-FU alone or the ODNs combined with 5-FU, and were analyzed for apoptosis by ELISA assay. Histograms represent fold difference in apoptosis level after setting the level of DLD1 control cells to 1.0. Also shown, Effectene alone as a control for the transfectant agent, and unexposed control cells. *, Significant difference in the apoptosis level by unpaired *t* test (refer to the text).

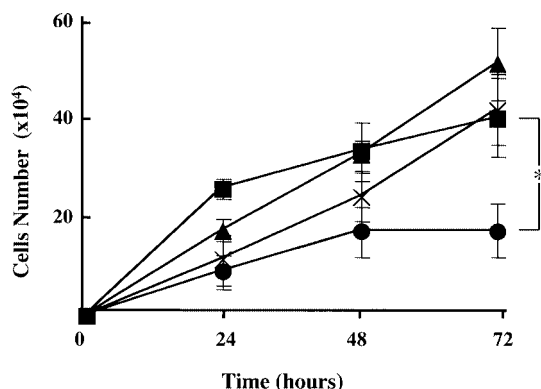


Fig. 6. Time-response curve showing the effect of AS1 on the cytotoxicity of 5-FU. DLD1 cells were exposed to 21 μ M 5-FU alone (cross), or 1 μ M AS1 alone (triangle), or 5-FU combined with AS1 (circle) or with its control NS1 (square) at the dose of 1 μ M, and the cell viability was determined by trypan blue exclusion assay after 24, 48 and 72 h of incubation. Results are shown as viable cell number. *, Significant difference in the viable cells number by unpaired *t* test.

combined with 5-FU (Fig. 5C), confirming the flow cytometric analysis. In addition, DNA fragmentation was assessed to confirm and quantify the level of apoptosis (Fig. 5D). We found a statistically significant difference (unpaired *t* test) between the levels of apoptosis of cells exposed to AS1 ODN and 5-FU when compared with cells exposed to AS1 ODN alone ($P=0.0003$), cells exposed to 5-FU and NS1 control ODN ($P=0.001$), and cells exposed only to 5-FU ($P<0.0001$). These studies confirm that AS1, by decreasing the Bcl-X_L levels, is able to significantly increase the rate of apoptosis induced by 5-FU.

Inhibition of Bcl-X_L protein sensitizes cells to 5-FU
 The ultimate goal of this research was to test the hypothesis that antisense oligonucleotides would be able to overcome the resistance of colon cancer cell lines to 5-FU. Because AS1 was able to increase the rate of apoptosis in a specific manner, we test the possibility that this increased apoptotic rate was translated into growth inhibition. Using the 5-FU IC₅₀ dose at 72 h,¹¹ AS1 ODN coupled with 5-FU decreased viable colon cancer cells by 40% more than did 5-FU alone or AS1 ODN alone (unpaired *t* test, $P<0.05$). Thus, although AS1 ODN alone is able to induce apoptosis, its combination with 5-FU increases the growth-inhibitory action of the 5-FU. Furthermore, incubation of 5-FU with AS1 ODN resulted in a significant growth inhibition of DLD1 cells when compared with 5-FU and NS1 control ODN, exposed to an equal dose of 5-FU (Fig. 6). This suggests a specific growth-inhibitory effect of AS1 ODNs.

DISCUSSION

A major obstacle in the treatment of solid tumors with conventional anticancer agents is the development of drug resistance, which remains a major obstacle to a successful therapeutic outcome. The hypothesis that failure to undergo apoptosis contributes to the development of resistance to anticancer agents has been the subject of extensive research.^{1,16} The susceptibility of a cell to apoptosis induction appears to be regulated by the relative expression levels and interactions among the Bcl-2 family of apoptosis regulators, as well as other molecules involved in the apoptotic pathway.² Bcl-2 and Bcl-X_L are two members of this family and they can work in a common pathway to inhibit cell death.

There are several lines of evidence supporting the idea that down-regulation of Bcl-X_L represents a biologically and clinically relevant approach to potentiate 5-FU-based chemotherapy. First, several routes are involved in 5-FU enzymatic conversion to the nucleotide, and loss or decreased activity of the enzymes involved in these routes may lead to resistance to the cytotoxic effects of 5-FU.¹⁷ However, Fisher *et al.*¹⁸ showed that resistance to apoptosis induction owing to overexpression of Bcl-2 may be another arbiter of cellular response to 5-FU. Second, we have shown here that Bcl-X_L overexpression is also able to induce resistance to 5-FU in Colo320 cells. This finding is consistent with previous work in other cell systems.^{3,10} Third, there is evidence for a differential activity of Bcl-X_L and Bcl-2 against chemotherapy-induced apoptosis. Simonian *et al.*¹⁴ have suggested that cells transfected with Bcl-X_L are more resistant to 5-FU than cells transfected with Bcl-2. Furthermore, 5-FU has been shown to induce phosphorylation of Bcl-2, which would lead to loss of the anti-apoptotic activity of Bcl-2.¹⁹ Finally, because anticancer drugs eradicate cancer cells by activating apoptosis, the choice of anticancer agents should take into account the levels of Bcl-X_L and Bcl-2 expressed by the tumor cells. In this respect, this study shows that colorectal cancer cells have strikingly elevated levels of Bcl-X_L rather than Bcl-2 protein. Our results are in accordance with the report by Krajewska *et al.*²⁰ that immunohistochemical methods revealed a reduced expression level of Bcl-2 and an increased Bcl-X_L level in colorectal adenocarcinomas. Therefore, colorectal cancer is representative of cell systems overexpressing Bcl-X_L protein rather than Bcl-2. Thus, Bcl-X_L seems to be a biologically and clinically relevant target to potentiate 5-FU-based chemotherapy. An important consequence of these results is that overexpression of proteins with apoptosis-inhibiting action, such as Bcl-X_L, could render colon cancer cells more resistant to anti-cancer drug therapy.

Against this background, we set out to determine whether ODNs directed against Bcl-X_L are able to enhance

the effects of 5-FU on the growth of human colon cancer cell lines. The identification of antisense oligonucleotides with the greatest activity remains rather empirical.^{6,7)} We therefore designed and tested several oligonucleotides to hybridize to coding and non-coding regions of Bcl-X_L mRNA. The screening showed that the most active antisense ODNs was the sequence that targeted the start codon of the Bcl-X_L mRNA. Because AS1 ODNs was the most potent antisense ODNs sequence using our methodology, and since antisense targeting the start codon of Bcl-X_L mRNA, has been successfully employed by others,^{8,21)} we chose to perform all the experiments with the AS1. This is not in accordance with the report of Taylor *et al.*,²²⁾ who identified another sequence (ISIS 16009) that was more potent in decreasing the Bcl-X_L expression. However, they also reported an antisense molecule, ISIS 15999, which targeted the start codon and showed that it was also able to down-regulate the expression of Bcl-X_L, although not as effectively as ISIS 16009.

To show that an antisense molecule works through sequence-specific mechanisms has proved to be a hard task, since antisense ODNs interact with several other molecules and non-specific effects are frequently seen.^{6,7)} Here, we observed a consistent and reproducible reduction of Bcl-X_L protein, which was dose-dependent. This is in accordance with previous reports of similar antisense ODNs sequence used in different cell systems.^{8,21)} Two different controls for the antisense ODNs did not modulate the Bcl-X_L expression level or DLD1 colon cancer cell line viability. Moreover, there were no alterations in the protein expression of β -actin, Bax or Bcl-2 after treatment with the ODNs. Together, these results provide strong evidence for a sequence-specific reduction of Bcl-X_L protein.

Apoptosis was analyzed in terms of three different parameters. Using these approaches we were able to show that AS1 ODNs could induce apoptosis and significantly sensitize the DLD1 colon cancer cell line to apoptosis induced by 5-FU. Indeed, inhibition of the endogenous levels of Bcl-X_L caused about a 30-fold increase of apoptosis in DLD1 cells, suggesting that high expression levels of Bcl-X_L in colon cancer cell lines have a protective effect under normal resting conditions. This would be critical for normal colorectal cells *in vivo*, because the lower digestive tract is continuously exposed to several types of noxious compounds. Such a protective function of Bcl-X_L has been observed in other cell systems using antisense ODNs.^{8,21,23)} More importantly, down-regulation of Bcl-X_L significantly increased the susceptibility of the colon can-

cer cell line to treatment with 5-FU. The 5- to 10-fold increase of apoptosis induced by 5-FU alone changed to a 55-fold increase by the combination of AS1 ODNs with 5-FU.

Having demonstrated that AS1 ODNs can induce increased apoptosis of colon cancer cells by reducing Bcl-X_L expression, we examined whether such reduction was associated with enhanced cell growth inhibition in response to 5-FU-based therapy. When DLD1 cells were exposed to the AS1 ODNs combined with 5-FU there was an increase of 40% in the efficacy of the 5-FU. The dose-response relationship and the absence of antitumor effects with sense and scrambled oligonucleotides control support the hypothesis that Bcl-X_L antisense can sensitize colon cancer cell line to 5-FU. In agreement with this, Arriola *et al.*²⁴⁾ have also reported a key role for antisense ODNs therapy against Bcl-X_L, rather than Bcl-2, in modulation of anticancer agent-induced apoptosis and chemosensitivity in human testicular germ cell tumors.

Several potential limitations must be analyzed in future studies. For example, cancer cells are likely to be resistant to drug therapy through multiple mechanisms. Indeed, we were unable to increase the apoptosis level beyond 60%, despite optimization of the methodology. Another important aspect is that Bcl-X_L is not overexpressed in some colorectal cancers. It is unlikely that AS1 ODNs would be useful in such a circumstance. Thus, studies examining the effects of modulating other pathways involved in colorectal cancer development and prognosis, such as p53-based gene therapy, are of obvious importance.

We have described antisense ODNs that can effectively down-regulate Bcl-X_L protein expression, thereby increasing the sensitivity of this cancer cell line to 5-FU. Our results have demonstrated that manipulation of the apoptotic pathway is feasible and synergistic to 5-FU, opening a way to overcome, at least partly, the resistance of solid tumors to conventional chemotherapy. Further pre-clinical evaluation is clearly warranted.

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