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ORIGINAL ARTICLE

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Bcl-2 antisense oligonucleotides chemosensitize human gastric cancer in a SCID mouse xenotransplantation model

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Abstract We used Bcl-2 antisense oligonucleotides (G3139) to chemosensitize human gastric cancer by downregulation of Bcl-2 expression in vivo. Oligonucleotides and cisplatin were administered systemically in a human gastric cancer SCID mouse model, and Bcl-2 expression, apoptosis, tumor size, and survival were assessed. Used alone, G3139 treatment led to downregulation of Bcl-2 and moderate tumor reduction compared to saline control. G3139 combined with cisplatin treatment markedly enhanced the antitumor effect of cisplatin (70% tumor size reduction vs. cisplatin alone), associated with increased apoptosis measured in tumor biopsy specimens. Combined treatment with G3139 and cisplatin prolonged survival of the tumor-bearing SCID mice by more than 50% without adding significant drug-related toxicity. Treatment with Bcl-2 antisense oligonucleotides is thus a promising novel approach to enhance antitumor activity of cisplatin or other drugs used in gastric cancer therapy and warrants further evaluation in clinical trials.

Keywords Gastric cancer · Antisense oligonucleotides · Bcl-2 · Chemoresistance · Apoptosis

Abbreviations *ASO:* Antisense oligonucleotides · *MM:* Mismatch · *RC:* Reverse control · *SCID:* Severe combined immunodeficient · *TUNEL:* Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

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Introduction

Gastric cancer is the second most common type of cancer worldwide [1, 2]. Although its incidence in the United States has been falling over the past decade, more than 16,000 persons per year die there of the consequences of malignant gastric neoplasia. In some Asian countries gastric cancer is an even more important health issue due to an incidence rate ten times higher than that in North America or Europe [1, 2, 3].

Surgical treatment is the therapy of choice for gastric cancer if disease is localized [4]. However, this malignancy is diagnosed at a nonresectable stage in more than two-thirds of patients. Extensive studies over the past 30 years have compared the benefits of various chemotherapeutic regimens in this group of patients. Despite intense efforts, response rates and total survival remain poor. Standard chemotherapeutic drugs such as 5-fluoruracil, cisplatin, mitomycin C, and combinations thereof are associated with a median survival of 6–9 months [1, 4, 5, 6]. Although new chemotherapeutic regimens are currently being evaluated, the treatment of gastric cancer still is a major challenge to modern cancer therapy [4, 7, 8].

Many of the chemotherapeutic drugs administered in the treatment of gastric cancer are known to exert their cytotoxic effects by induction of apoptosis [9]. Apoptosis is a highly conserved cellular process allowing cells in response to a variety of stimuli to activate preformed enzymes that culminates in the activation of nucleases and cysteine aspartic acid specific proteases (caspases) responsible for the degradation of cellular proteins and ultimately leading to decay of the affected cells [10, 11]. During the past decade apoptosis has become a major research focus of experimental oncology since it became increasingly clear that most anticancer drugs mediate their action to tumor cells through this type of programmed cell death [9]. The rate or susceptibility of cells to undergo apoptosis after a chemotherapeutic stimulus is influenced by the expression of certain oncogene and tumor suppressor gene products [11]. One of these proteins is Bcl-2, first described in B-cell lymphoma in 1985 [12]. It is the founding member of a family of proteins consisting of pro- (e.g., Bax, Bak, Bad) and antiapoptotic (e.g., Bcl-xl, Mcl-1) factors [13, 14]. While the exact mechanism of antiapoptotic Bcl-2 effects has not yet been fully elucidated, the basic antiapoptotic properties of Bcl-2 and its ability to inhibit tumor cell apoptosis induced by numerous chemotherapeutic agents, radiation, and other stimuli are well known [11, 13].

The Bcl-2 protein has been found to be expressed in up to 72% of cases of gastric cancer [15, 16, 17]. The role of Bcl-2 in the tumor biology of gastric cancer and its prognostic significance are still under investigation. Several studies have reported a significantly lower proportion of apoptotic cell death in Bcl-2 positive gastric tumors than in their Bcl-2 negative counterparts [18, 19]. Chemoresistance and total survival of gastric cancer patients have recently been linked to Bcl-2 expression in tumors expressing both Bcl-2 and the proapoptotic protein Bax [20].

Based on the available evidence, Bcl-2 appears to be an attractive target for treatment strategies designed to enhance the chemosensitivity of gastric cancer. A possible clinical treatment approach to modulating the expression of oncogene products is the systemic administration of antisense oligonucleotides (ASO). ASO are modified single-strand stretches of nucleotides capable of inhibiting protein expression by complexing with the complementary target mRNA and thus preventing their translation. ASO have been shown to be well tolerated, valuable, and effective tools for downregulating a specific target protein also in vivo [21, 22, 23, 24, 25, 26].

In this study we used Bcl-2 ASO (G3139) to downregulate Bcl-2 protein expression in human gastric cancer cells in vitro and in vivo. This downregulation chemosensitized human gastric tumors to a subsequent treatment with cisplatin in a severe combined immunodeficient (SCID) mouse xenotransplantation model and forms the basis for further evaluation of this strategy in patients with Bcl-2 positive gastric cancer.

Materials and methods

Cell lines and cell culture

The human gastric cancer cell lines Kato-III and N87 were obtained from American Type Culture Collection. Cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic mix (Gibco BRL) in a humidified 5% CO_2–95% ambient air atmosphere at 37°C. For in vitro experiments cells were seeded at a density of 1.5×10^5 in six-well plates (BD, Franklin Lakes, N.J., USA). After 24 h cells were incubated with the indicated oligonucleotide and cisplatin concentrations.

Oligonucleotides

Phosphorothioate oligodeoxynucleotides corresponding to the human Bcl-2 translation initiation site were obtained from Genta (Berkeley Heights, N.J., USA). The antisense sequence was 5′-TCTCCCAGCGTGCGCCAT-3′ (AS, G3139, chemical name augmerosen). Two base Bcl-2 antisense mismatch – (MM, 5′-TCTCCCAGCATGTGCCAT-3′, G4126) and reverse control phosphorothioate oligonucleotides (RC, 5′-TACCGCGTGCGACCCTCT-3′, G3622) were used as controls. Oligonucleotides were solved in saline solution (Sal).

Experimental animals and tumor treatment in vivo

Pathogen-free female C.B-17 *scid/scid* mice 4–6 weeks old, tested for leakiness, were obtained from Bomholtgard Breeding and Research Center (Ry, Denmark). Animals were housed in microisolator cages in laminar flow racks and received autoclaved food and water ad libitum. SCID mice (six mice per group) were injected subcutaneously into the left lower flank with 1.5×10^7 N87 human gastric cancer cells resuspended in 200 µl phosphate-buffered solution. Seven days later mice were anesthetized, and miniosmotic pumps (Alzet 2004, Alza, Moutain View, Calif., USA) filled with oligonucleotides in saline solution or saline as vehicle control were implanted subcutaneously into a paraspinal pocket [21]. Implanted pumps released their contents at a rate corresponding to 10 mg/kg per day over a period of 28 days.

Mice were treated with cisplatin (Ebewe, Unterach, Austria) administered intraperitoneally at the time point and concentration indicated. SCID mice were evaluated for tumor growth and total survival. Tumor volume was calculated as previously described [27]. Mice were killed after tumor progression had caused terminal disease (judged by an independent research animal veterinarian blinded as to the treatment groups, at least 1.5 cm^3 tumor volume). The study was approved by University of Vienna animal care committee.

Western blot analysis

Western blotting for Bcl-2, Bcl-xl, and actin was performed as previously described [21]. In brief, whole-cell extracts of cultured

cells or minced human gastric tumors grown in SCID mice were prepared using 0.14 M NaCl, 0.2 M triethanolamine, 0.2% Na-deoxycholate, and 0.5% Nonident P-40, supplemented with protease inhibitors (all Sigma, St. Louis, Mo., USA). Of the total protein 15 µg was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted to polyvinylidene fluoride membranes (Tropix, Bedford, Mass., USA). The membranes were blocked for 1 h in 0.2% I block (Tropix) in phosphate-buffered solution and then incubated with monoclonal antibodies recognizing Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and Bcl-xl (BD, Franklin Lakes, N.J., USA). Second antibody incubations were carried out using goat anti-mouse or goat anti-rabbit conjugated to alkaline phosphatase (Tropix) and reactive bands were detected by chemiluminescence (CSPD substrate, Tropix). An anti-actin antibody (Sigma) was used as an internal control to demonstrate equal protein loading in each lane. The expression levels of Bcl-2 and actin protein were quantified by densitometry of autoradiogramms with a Herolab EASY RH densitometer (Herolab, Wiesloch, Germany) and the EASY Win32 software (Herolab). Signal strength of each Bcl-2 signal was normalized to actin and the ratios between Bcl-2 protein in antisense oligonucleotide-treated extracts and control tumor extracts were calculated [28]. Bcl-2 downregulations below 20% were regarded as not significant.

Histological evaluation and TUNEL staining of tumors

On day 28 two mice per group were killed, and organs (brain, heart, lungs, liver, spleen, kidneys) and tumors were fixed in neutral buffered formalin (7.5% formalin in 0.08 M sodium phosphate, pH 7.4) for 24 h. Tissue was then embedded in paraffin, cut into sections of 2 µm, and routinely stained with hematoxylin-eosin for morphological analysis. Multiple tumor sections were obtained (minimum of five). Only comparable sections of the outer layers of the tumor were analyzed. Additionally, sections with evidence of necrotic areas or physically damaged cell morphology in hematoxylin and eosin staining were excluded from further analysis.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) immunohistochemistry was performed in tumor tissue according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). In brief, tissue sections were dewaxed, incubated with proteinase K, and permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate on ice. Phosphate-buffered solution rinsed slides were then treated with the TUNEL reaction mixture in a humidified chamber at 37°C. Sections were counterstained with 4'-6'-diamidino-2-phenylindole nuclear stain, and apoptotic index was determined by fluorescence microscopy blinded as to the treatment groups.

Statistical analysis

Statistical significance of differences in tumor weight were calculated by using the Mann-Whitney *U* test. Survival curves were computed using the Kaplan-Meier method. Multiple comparisons were calculated using one-way analysis of variance, and posttests among treatment groups were performed using the Scheffé test (Statistica release 5.1, StatSoft, Tulsa, Okla., US). *P* values less than 0.05 were considered to be statistically significant.

Results

The two gastric cancer cell lines Kato-xIII and N87 are known to express the antiapoptotic protein Bcl-2. Using Bcl-2 ASO (G3139) at nanomolar concentrations in the presence of the uptake enhancer lipofectin, we observed in both cell lines a pronounced reduction in Bcl-2 levels in vitro. Bcl-2 ASO at a concentration of 200 nM for 48 h caused a twofold reduction in Bcl-2 protein compared to saline controls in Kato-III and N87 cells (Fig. 1; saline 100±7.3%, ASO 51±10.6% test/control, $P<0.02$; for KATO III data not shown). There was no or only a minor reduction in Bcl-2 protein expression in cells incubated in the presence of the same concentration of control oligonucleotides (MM 85±3.4% test/control; RC 103±5.4% test/control).

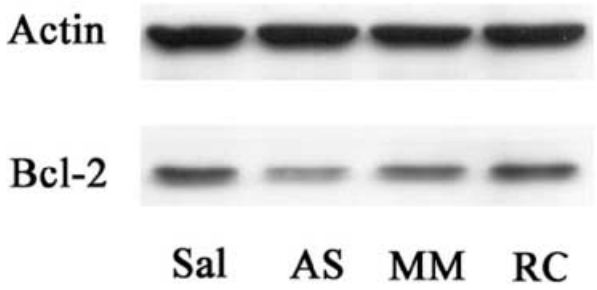

Fig. 1 Downregulation of Bcl-2 in vitro. Western blot of N87 treated for 48 h with 200 nm oligonucleotides in the presence of uptake-enhancing Lipofectin. *Sal* Saline; *AS* antisense oligonucleotides; *MM* mismatch oligonucleotides; *RC* reverse control oligonucleotides. This experiment was repeated four times, and data from a representative experiment are shown

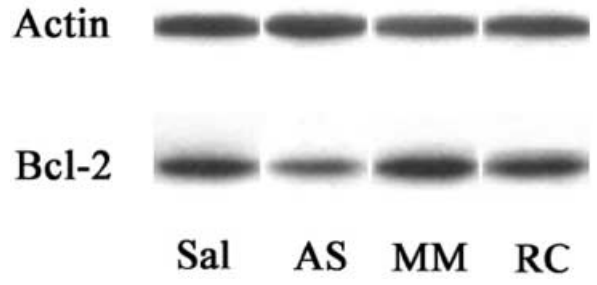

Fig. 2 Downregulation of Bcl-2 in vivo after 14 days by continuous administration of Bcl-2 antisense oligonucleotides. *Sal* Saline; *AS* antisense oligonucleotides; *MM* mismatch oligonucleotides; *RC* reverse control oligonucleotides. A representative blot is presented (n=3 mice per group)

To test whether Bcl-2 ASO also downregulate Bcl-2 expression in vivo, Bcl-2 ASO (G3139), control oligonucleotides (MM/RC), or saline as vehicle control were administered via continuous infusion delivered by miniosmotic pumps in a human gastric cancer SCID mouse xenotransplantation model. Since Kato-III cells were not tumorgenic in SCID mice (data not shown), we focused on N87 for in vivo.

In a pilot study (n=3 mice per group) oligonucleotides were administered at a concentration of 10 mg/kg per day for 14 days, a dose previously shown to be effective in other tumor models [28, 29]. After 14 days mice were killed, and tumor biopsy specimens were assessed for Bcl-2 expression. Human gastric cancer xenografts of all mice treated with ASO showed significant reductions in Bcl-2 expression by approximately one-third compared

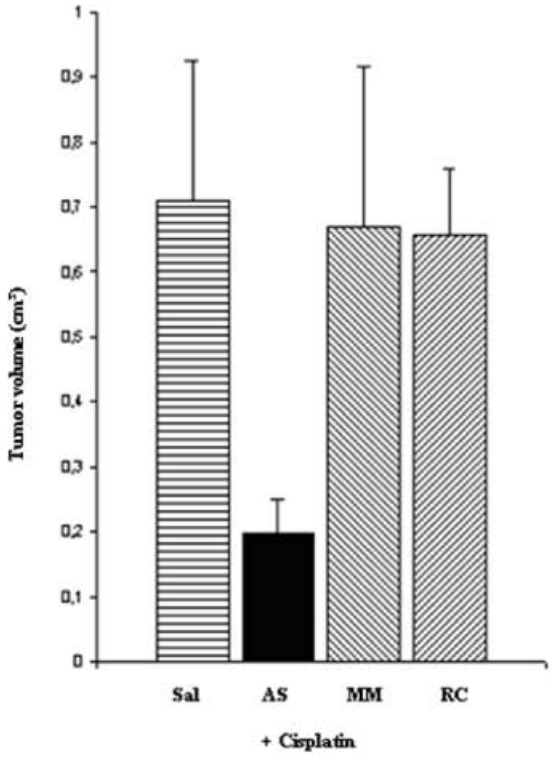

Fig. 3 Bcl-2 antisense oligonucleotides chemosensitize human gastric tumors in vivo. SCID mice (*n*=6) injected with N87 gastric cancer cells treated with saline (*Sal*) or with antisense (*AS*), two-base mismatch (*MM*), or reverse control (*RC*) oligonucleotides at a dose of 10 mg/kg per day. On day 7 an intraperitoneal bolus injection of cisplatin was administered to all groups. Mean tumor volume in cubic centimeters; *Bars* SD

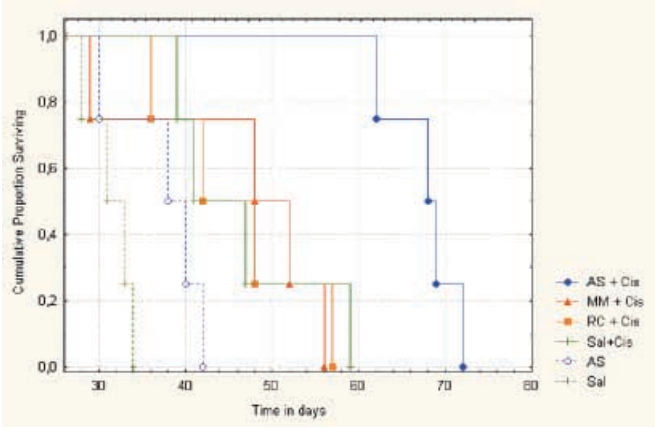

Fig. 5 Bcl-2 antisense oligonucleotides in combination with cisplatin prolong total survival in vivo. SCID mice injected with N87 gastric cancer cells received for 28 days saline (*Sal*) or antisense (*AS*), mismatch (*MM*), or reverse control (*RC*) oligonucleotides at a dose of 10 mg/kg per day. On day 7 mice were treated with an intraperitoneal bolus injection of 9 mg/kg cisplatin (*Cis*). Mice were killed when tumor progression had caused terminal disease

to tumors of mice which received either saline or control oligonucleotide treatment (Fig. 2) without apparent toxicity to the animals (saline 100±7.1%, ASO 65±6.6% test/control, saline vs. ASO $P<0.03$; MM 104±11.0% test/control, RC 98±9.2% test/control). Bcl-xl expression remained unchanged in all groups (data not shown).

Following the observation of a Bcl-2 downregulation by ASO in vivo, the effect on tumor volume of ASO treatment for 28 days at a concentration of 10 mg/kg per day alone and in combination with cisplatin was then evaluated in the next experiment (*n*=6 mice per group). Single-agent Bcl-2 ASO therapy resulted in moderately less tumor volume than saline control (ASO 0.53±0.212 cm^3, saline 0.93±0.20 cm^3, $P<0.019$). In contrast, the combined treatment with G3139 Bcl-2 ASO plus an intraperitoneal bolus of 9 mg/kg cisplatin on day 7, a dose well tolerated in SCID mice [30], led to a dra-

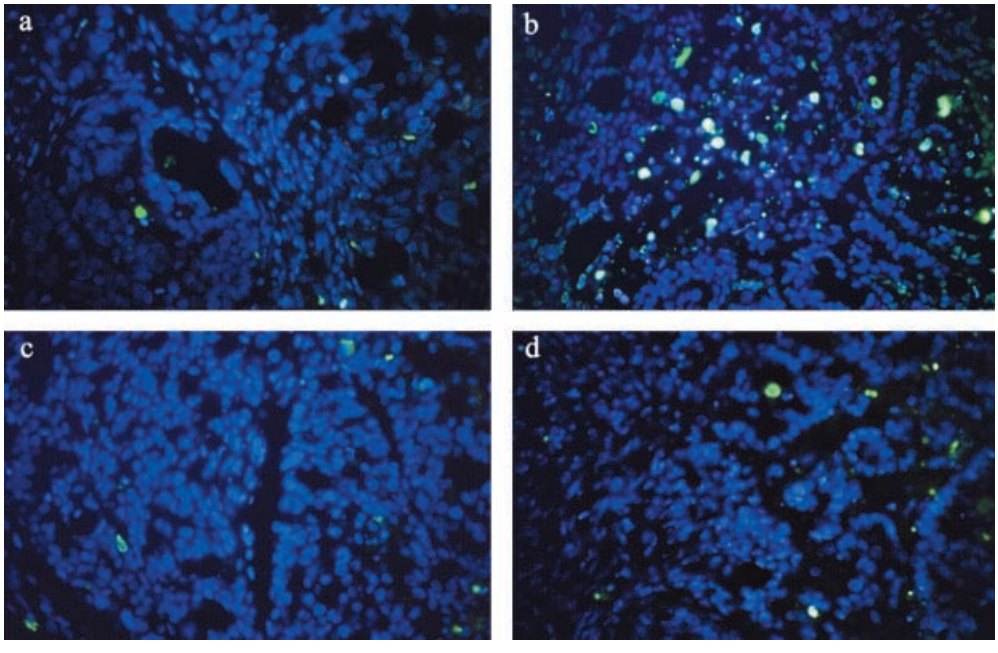

Fig. 4a–d TUNEL staining of gastric tumors grown in SCID mice (apoptotic cells appear *fluorescent green*; counterstain is 4'-6'-diamidino-2-phenylindole). Representative photographs of N87 tumors treated with cisplatin and saline (**a**) or antisense (**b**), mismatch (**c**), or reverse control (**d**) oligonucleotides (**d**) for 28 days. Original magnification ×200

matic reduction in tumor volume compared to control treatments. After 14 days of continuous infusion of Bcl-2 ASO and single bolus administration of cisplatin on day 7, mice showed more than 70% reduction in mean tumor volume (ASO+cisplatin 0.19±0.05 cm^3) compared to mice treated with cisplatin plus either control oligonucleotides or saline (MM+cisplatin 0.67±0.24 cm^3, RC+cisplatin 0.66±0.10 cm^3; saline+cisplatin 0.71±0.21 cm^3; Fig. 3). These differences were all highly significant ($P<0.006$). The treatment combinations with oligonucleotides plus cisplatin led to no apparent additional toxicity to the mice than cisplatin alone.

To investigate whether this chemosensitization effect by Bcl-2 ASO was linked to a higher rate of apoptotic cell death in tumors xenografts, two mice per group were killed on day 28, and tumors were examined for apoptotic cell death by TUNEL staining. Single-agent Bcl-2 ASO treatment revealed a trend towards increased apoptosis that was statistically not significant (data not shown). However, the combined administration of Bcl-2 ASO plus cisplatin resulted in an approximately threefold increase in the rate of apoptotic tumor cells from 1.9% (saline+cisplatin group) to 6.7% (ASO+cisplatin group; MM+cisplatin 2.2%, RC+cisplatin 2.6%; Fig. 4).

In view of the chemosensitization effect of Bcl-2 ASO observed by measurement of tumor volume, we additionally investigated the influence of Bcl-2 ASO with and without cisplatin on end-point survival due to tumor burden in the four mice per group kept alive on day 28 (Fig. 5). Median survival in the antisense-only treated group was 39 days (37.5±5.3 days), compared to 32 days in the saline group (31.5±2.6 days; $P>0.93$). Compared to the antisense-alone treated group, cisplatin alone (median 44, mean 46.5±9.1 days) was also not statistically significant ($P>0.74$). However, the combination of Bcl-2 ASO and cisplatin resulted in significantly longer survival than with cisplatin alone or with any combination of control oligonucleotides and cisplatin (all $P<0.037$; Fig. 5). Although four animals per group is certainly a small number and limits the strength of the findings, the median survival of mice treated with Bcl-2 antisense and cisplatin was 69 days (mean 67.6±4.2 days), corresponding to a 56% improvement in median survival compared to treatment with cisplatin alone. Survival of animals receiving control oligonucleotides (MM/RC) and cisplatin did not differ statistically from cisplatin alone ($P>0.9$), with a median survival of 50 days (MM+cisplatin, 46.3± 11.9 days) and 45 days (RC+cisplatin, 45.8±9.0 days).

Discussion

To date, there is no satisfactory treatment for advanced gastric cancer. The poor prognosis of this malignancy is at least in part due to resistance to a variety of conventional and novel chemotherapeutic regimens in which the mechanism of action has been linked to apoptosis. In this study we observed a clear chemosensitizing effect of Bcl-2 ASO to cisplatin for gastric cancer in a SCID-hu mouse model, measured by antitumor response, apoptosis, and survival related to tumor burden. Both in vitro and in vivo experiments demonstrated clear downregulation of the Bcl-2 target protein by the Bcl-2 ASO, effects that were sequence specific and were not seen with two control oligonucleotides.

The observation that Bcl-xl, another antiapoptotic member of the Bcl-2 family with 74% homology to the Bcl-2 protein [13, 14], was not altered by the Bcl-2 ASO treatment (data not shown) further supports the specific antisense mechanism of the Bcl-2 ASO drug investigated. Furthermore, there was no observed reciprocal upregulation of Bcl-xl expression to Bcl-2 antisense downregulation, a compensatory mechanism reported for other malignancies [13].

In gastric cancer Bcl-2 expression coincides with a lower fraction of apoptosis in tumor biopsy specimens [31]. Reflecting this inverse correlation between apoptotic fraction and Bcl-2 expression, Bcl-2 downregulation by ASO resulted in a strikingly higher rate of apoptosis after cisplatin treatment than in any control group. These findings can be interpreted as a clear sign of facilitated apoptotic cell death. The absolute amount of Bcl-2 reduction by antisense strategies may not be the only crucial factor determining the possible chemosensitization. Since it was not necessary to block Bcl-2 expression completely to observe the effects demonstrated, it seems rather important to shift the balance/ratio between key pro- and antiapoptotic factors towards apoptosis [9].

This concept is supported by data presented in a recently published study examining two breast cancer cell lines with different levels of endogenous Bcl-2 expression. The relative degree of Bcl-2 downregulation, possibly coupled with the baseline ratio of Bcl-2 to other pro- or antiapoptotic proteins, appeared more important than reductions in the absolute amount of Bcl-2 in dictating Bcl-2 antisense effects [32]. Furthermore, it is important to note that even transient downregulation of a target protein obtained by repeated bolus injections of ASO chemosensitized tumors in vivo whereas we used continuous administration by miniosmotic pumps, resulting in a decrease in Bcl-2 expression after 14 days [25, 26].

In this study we focused on the chemosensitization potential and not the single agent activity of the Bcl-2 ASO. Therefore we did not use control oligonucleotides (MM/RC) without the combination of cisplatin as control groups in the chemosensitization experiment. In the pilot study we found no sign of antitumoral effects in mice treated with control oligonucleotides alone compared to saline control in contrast to ASO. Furthermore, experience from prior experiments in other tumor entities showed no effect of either control oligonucleotides on tumor growth [21, 25, 26, 28].

In gastric cancer there is evidence that Bcl-2 may be one decisive link with chemoresistance and therapeutic outcome [20]. Shiguzu et al. demonstrated that Bcl-2 expression in gastric cancer is correlated with poor outcome if the tumors also express the proapoptotic protein Bax which heterodimerizes with Bcl-2. Notably, in their

study nonresponders to a combined treatment of 5-fluoruracil and cisplatin were twice as often positive for Bcl-2 expression as responding patients. For these patients chemosensitization by Bcl-2 ASO might be a promising approach to overcome chemoresistance.

In addition to a reduction in the volume of gastric cancer tumor xenotransplants by about 70%, the combination of Bcl-2 antisense plus chemotherapy more than doubled the survival of saline-treated animals, an effect seen with no other treatment combination. Moreover, our study showed a more than 50% prolonged survival, from 44 to 69 days, by combination treatment than with cisplatin alone. This effect, observed in four animals per group, was seen even though all treatment was stopped after 4 weeks.

In agreement with other Bcl-2 antisense studies in solid tumor models, Bcl-2 ASO alone led to no major improvement in total survival, supporting the concept of using Bcl-2 ASO in combination with chemotherapy as a chemosensitizer rather than as a single agent. 5-Fluoruracil, the most established single-agent drug in palliative chemotherapeutic gastric cancer care, showed no antitumor effects in our pilot experiments (data not shown). Furthermore, the tumor response of 5-fluoruracil is reported to be inadequately predicted in SCID mouse models for reasons not yet fully understood [33]. We therefore chose cisplatin rather than 5-fluoruracil, a known powerful inducer of apoptosis also frequently used in the palliative treatment of gastric cancer patients [6, 8].

Recently it has been reported that ASO in addition to their antisense mechanism also have immune stimulatory effects due to CpG motifs (for a review see Krieg et al. [34]). The G3139 Bcl-2 antisense used in this study contains two CpG motifs. However, immune stimulation via CpG does not appear to be a major mechanism contributing to the antitumor effects observed. Immune stimulation by CpG oligonucleotides requires NK cell activity [34]. Carpentier et al. [35] demonstrated in a well designed CpG study that NK cell depletion by monoclonal antibody anti-NK1.1 abrogates antitumoral effects of CpG oligonucleotides. The ASO used in our experiments have been examined in several other tumor models without any signs of immunostimulatory effects. Waters et al. [36] and Klasa et al. [25] tested the identical antisense oligonucleotide G3139 in nonobese diabetic SCID mice (SCID mice with a relative NK cell deficiency) and perforin deficient SCID mice (NK cells present, but no lysis of cells possible resulting in functional NK cell deficiency) in a lymphoma model and observed no difference in antitumoral effects in SCID mice (functional NK cells). Furthermore, major CpG effects require peritumoral injection of CpG oligonucleotides, and only minor effects on primary tumors have been observed when CpG oligonucleotides were administered systemically as the ASO in this study [35]. In addition, it is known that methylation of oligonucleotides, which is reported to abolish or severely reduce immune stimulation by CpG motifs [34], does not alter the antitumor potential of G3139 Bcl-2 antisense in other xenotransplantation systems [21].

ASO, including the Bcl-2 ASO drug G3139 used in this study, have been shown to be well tolerated in ongoing phase I/II clinical trials [22, 23]. Furthermore, it has been demonstrated in stage IV melanoma patients that G3139 reduces the targeted protein within solid tumor lesions, facilitates apoptosis, and allows chemosensitization to dacarbazine therapy with clinical responses in patients with advanced disease who failed to respond to other treatment regimes [24].

In conclusion, Bcl-2 antisense treatment with G3139 chemosensitizes cisplatin treatment of human gastric cancer, leading to better response and survival than in all control groups tested. The data provide further evidence that Bcl-2 expression in gastric cancer is a factor responsible for resistance and poor treatment outcome. Moreover, these findings suggest further evaluation of Bcl-2 ASO to improve the treatment provided by cisplatin, and possibly other anticancer drugs used for therapy of patients with gastric cancer.

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