Mcl-1 Is a Novel Therapeutic Target for Human Sarcoma: Synergistic Inhibition of Human Sarcoma Xenotransplants by a Combination of Mcl-1 Antisense Oligonucleotides with Low-Dose Cyclophosphamide

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ABSTRACT

Purpose: Little is known about the role that Mcl-1, an antiapoptotic Bcl-2 family member, plays in solid tumor biology and susceptibility to anticancer therapy. We observed that the Mcl-1 protein is widely expressed in human sarcoma cell lines of different histological origin (n = 7). Because the expression of antiapoptotic Bcl-2 family proteins can significantly contribute to the chemoresistance of human malignancies, we used an antisense strategy to address this issue in sarcoma.

Experimental Design: SCID mice (n = 6/group) received s.c. injections of SW872 liposarcoma cells. After development of palpable tumors, mice were treated by s.c.-implanted miniosmotic pumps prefilled with saline or antisense or universal control oligonucleotides (20 mg/kg/day for 2 weeks). On days 2, 6, and 10, mice were treated with low-dose cyclophosphamide (35 mg/kg i.p) or saline control. During the experiments, tumor weight was assessed twice weekly by caliper measurements. On day 14, animals were sacrificed. Tumors were weighed and fixed in formalin for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling analysis.

Results: Mcl-1 antisense oligonucleotides specifically reduced Mcl-1 protein expression but produced no reduction in tumor weight compared with saline-treated control animals. Cyclophosphamide monotherapy caused only modest tumor weight reduction compared with saline control. However, use of Mcl-1 antisense oligonucleotides combined with cyclophosphamide clearly enhanced tumor cell apoptosis and significantly reduced tumor weight by more than two-thirds compared with respective control treatments.

Conclusion: A combination of Mcl-1 antisense oligonucleotides with low-dose cyclophosphamide provides a synergistic antitumor effect and might qualify as a promising strategy to overcome chemoresistance in human sarcoma.

INTRODUCTION

Sarcoma comprises several subtypes with different clinical behaviors and in general possesses a high metastatic potential. Depending on various factors, such as disease stage, location, and tumor grade, single or combined treatment strategies consisting of surgery, radiotherapy, and/or chemotherapy are applied (1–3). Unfortunately, the clinical outcome in advanced sarcoma is still disappointing (4–6), and the management of patients with advanced and recurrent disease is often merely palliative. One of the most frequently used chemotherapeutics in the treatment of human sarcoma is cyclophosphamide (CPA; Refs. 7–9). However, even with CPA response rates do not exceed 28% (7).

The Mcl-1 protein plays an important role in the development of various malignancies and is expressed in a wide variety of tissues and neoplastic cells (10–12). In addition to its antiapoptotic effects, Mcl-1 has also been shown to be involved in the regulation of cell-cycle progression (13). Compared with other members of the antiapoptotic Bcl-2 protein family, Mcl-1 is unique in its rapid and readily inducible expression after exposure to cytokines or growth factors (12, 14, 15). In contrast to Bcl-2 and Bcl-xL, Mcl-1 contains a 180-amino-acid-long NH2-terminal segment encoding a PEST (proline, glutamate, serine, and threonine) sequence (16), which may account for its short half-life of 1–2 h (15–17). Moreover, the intracellular distribution of Mcl-1 appears to be more widespread than Bcl-2: in addition to its predominant localization at mitochondrial membranes, Mcl-1 is also distributed in a variety of nonmitochondrial compartments (10, 18, 19).

To date, the mechanism of Mcl-1 antiapoptotic activity has not been clearly established. In HL-60 cells, for example, an increase of Mcl-1 expression has been associated with inhibition of apoptosis by down-regulation of cell-damage-induced mito-
chondrial cytochrome c release (20). Further studies will be required to gain detailed insights into the function Mcl-1 might play in the apoptotic machinery.

However, the role of Mcl-1 expression in supporting cell survival is well established and has been studied in various cell systems. Human neutrophils express neither Bcl-2 nor Bcl-xL, but they do express Mcl-1 (21). On shutdown of Mcl-1 expression, this prosurvival gene product is rapidly depleted, resulting in acceleration of apoptosis of human neutrophils (21). Similarly, down-regulation of Mcl-1 by antisense oligonucleotides causes the rapid entry of differentiating human myeloblastic leukemia cells (U937) into apoptosis (22). Mcl-1 also seems to be crucial for the survival of myeloma cells. Mcl-1 treatment triggers a pronounced decrease in viability of myeloma cells tested. Overexpression of Mcl-1 in Chinese hamster ovary (CHO) cells (23, 24) and murine myeloid progenitor (FDC-P1) cells (25) has been shown to delay apoptosis induced by miscellaneous stimuli. Tang et al (26) reported that in human melanoma, up-regulation of Mcl-1 is associated with malignant transformation. Furthermore, increased Mcl-1 and Bcl-xL levels are observed in thin primary melanomas as well as in metastatic malignant melanoma, but not in benign nevi. Taken together, these results indicate that Mcl-1 may be a key determinant in ensuring survival in different tumor entities by prolonging cell viability under various cytotoxic conditions (25).

Interest in novel strategies influencing apoptotic cell death of human sarcoma has increased significantly over the last few years. For example, cell survival of chondrosarcoma was shown to be inhibited by induction of apoptosis through the use of ligands to peroxisome proliferator-activated receptor-γ (27). Treatment of chemoresistant osteosarcoma cells by a combination of Apo2L/TRAIL with clinically used anticancer drugs resulted in a significant increase in apoptotic cell death (28). Apoptosis and cell cycle arrest in osteosarcoma cells can also be induced by the plant steroid diosgenin (29) or by selenium (30). Transfection of soft-tissue sarcoma cells with mdm2-antisense or wild-type p53 resulted in radiosensitization and increased apoptosis (31).

Antisense oligonucleotides (ASOs), chemically modified stretches of single-stranded DNA, are pharmacologically potent inhibitors of the expression of disease-related proteins (32–37). They are designed to bind to their complementary mRNA sequences once they enter cells, thereby inhibiting expression of the encoded proteins (38). Antisense strategies using mono- or bispecific ASOs against Bcl-2, Bcl-xL (39–41), and Mcl-1 (42) have shown promise as treatment strategies for various human malignancies. Previously, our group reported the chemosensitizing effects of Bcl-2 ASOs in human melanoma both preclinically and in a recently completed Phase II trial (32, 43).

In the present study, we show that Mcl-1 ASOs decreased Mcl-1 protein levels in human sarcoma xenotransplants, resulting in an enhanced susceptibility toward low-dose CPA treatment.

**MATERIALS AND METHODS**

**Cell Lines and Culture.** The human sarcoma cell lines used for this study were obtained from the American Type Culture Collection (Manassas, VA) and Clontech (Palo Alto, CA) and were maintained according to American Type Culture Collection and Clontech recommendations.

**Oligonucleotides and Transfection.** 2′-O-Methyl-ethylidynechimeric phosphorothioate ASOs were kindly provided by Isis Pharmaceuticals (Carlsbad, CA). The sequence of the Mcl-1 ASO (ISIS 20408) was 5′-TTGGCCTTGTGCCTTGGCG-3′. The universal control oligonucleotide (UC, ISIS 29848) used in these studies was synthesized as a mixture of A, G, T, and C bases so that the resulting preparation contained an equimolar mixture of all possible 4 to the 19th nucleotides oligonucleotides. The oligonucleotide chemistry of ISIS 29848 is identical to that of ISIS 20408. For transfection, 300,000 cells were seeded in a 75-cm² plate 24 h before the oligonucleotide treatment. Oligonucleotides were complexed with lipofectin (Life Technologies, Paisley, United Kingdom) in antibiotic-free medium without serum as described by the supplier. Subsequently, cells were incubated for 4 h with 200 nm oligonucleotides (10 µg/ml lipofectin) in antibiotic-free medium without serum. After being washed with DMEM, the cells were cultured in complete medium containing serum.

**Quantification of Cell Number.** For cell proliferation experiments, cells were seeded in 24-well plates (20,000 cells/well) and transfected with oligonucleotides as described above. After 4 h of incubation with the indicated oligonucleotides (200 nm), cells were cultured in complete medium containing serum with or without staurosporine (1, 2, 5, and 10 ng/ml; Sigma, St. Louis, MO). Because CPA is a cancer produrg requiring bioactivation in vivo (44, 45), we studied the chemosensitization potential of Mcl-1 ASO in vitro by use of staurosporine, a well-established experimental apoptosis inducer (46, 47). We determined cell numbers by quantifying cellular DNA after RNA digestion, using a CyQuant Cell Proliferation Assay kit (Molecular Probes, Leiden, the Netherlands) according to the manufacturer’s instructions. Cell culture plates were scanned with a Victor² 1420 Multilabel Counter (Wallac, Turku, Finland) adjusted to FITC settings.

**Western Blot Analysis.** Cell extracts were prepared in lysis puffer containing 0.14 M NaCl, 0.4 M triethanolamine, 0.2% sodium deoxycholate, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 4.0 µg/ml aprotinin, and 4.0 µg/ml leupeptin. The amount of soluble proteins was quantified by modified Bradford analysis (Bio-Rad, Richmond, CA). Total lysates (15 µg/lane) were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with anti-Mcl-1, anti-Bcl-2 (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-xL (Becton Dickinson, Franklin Lakes, NJ), or anti-β-tubulin antibody (Sigma). Alkaline phosphatase-conjugated goat antimouse and goat antirabbit immunoglobulins (Tropix, Bedford, MA) were used for secondary reactions. Reactive bands were visualized by chemiluminescence using CSPD as substrate (Tropix). Protein expression levels were quantified by densitometry of autoradiograms with an Ultrascan XL densitometer (Pharmacia, Uppsala, Sweden).

**Evaluation of Oligonucleotide Effects in Vivo.** Pathogen-free female C.B.-17 scid/scid (SCID) mice (4–6 weeks of age; Harlan Winkelmann, Borchen, Germany) were randomly assigned to experimental groups of six animals each. SCID mice received s.c. injections containing 1 × 10⁷ SW872 human human.
liposarcoma cells in the lower left flank. Five weeks after injection, when animals had developed sarcomas of similar size (nODULES ∼5 mm in diameter), treatment was initiated. Animals were anesthetized, and miniosmotic pumps (Alzet 2002; Alzet, Palo Alto, CA) pre-filled with saline, ASO, or UC were inserted s.c. into a paraspinal pocket (day 1). Implanted pumps released their contents by continuous infusion at a rate of 20 mg/kg/day over a period of 2 weeks. Mice were treated with CPA (35 mg/kg i.p.) on days 2, 6, and 10, in principle as reported previously (48). During the experiments, tumor weight was assessed twice a week by caliper measurement as described previously (49). On treatment day 14, all animals were sacrificed. Tumors xenotransplants were weighed and fixed in formalin for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis. Tumors xenotransplants were weighed and fixed in formalin for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis.

Apoptosis Assay and Immunohistochemistry. The histochemical detection of apoptosis in formalin-fixed sections was performed by TUNEL assay using fluorescein-dUTP (Boehringer Mannheim, Mannheim, Germany). Tissue sections were counterstained with 4',6-diamidine-2'-phenylindole dichloride (Roche Diagnostics, Vienna, Austria). The expression of Mcl-1 and cleaved caspase 3 in vivo was evaluated by immunohistochemistry with an anti-Mcl-1 antibody (Santa Cruz Biotechnology) or an anti-cleaved-caspase 3 antibody (New England Bio Labs, Beverly, MA) and a DAKO LSAB 2 System using diaminobenzenide as a chromogen.

Statistical Analysis. Statistical significance of differences in tumor weight among treatment groups was calculated by one-way ANOVA and the Bonferroni test for post hoc testing (SPSS 10.7; SPSS Inc., Chicago, IL). P values <0.05 were considered statistically significant.

RESULTS
Specific Reduction of Mcl-1 Protein in Human Sarcoma by ASOs in Vitro. We first examined Mcl-1 expression in a variety of different human sarcoma cell lines. As demonstrated by Western blot analysis, all seven human sarcoma cell lines evaluated were found to express the antiapoptotic protein Mcl-1 (Fig. 1A). When we used the Mcl-1 ASOs at nanomolar concentrations in the presence of the uptake enhancer lipofectin, we observed a significant reduction in Mcl-1 protein levels in vitro (Fig. 1B and C). Twenty-four h after treatment, the ASOs reduced Mcl-1 levels in SW872, HT1080, and SW1353 cells relative to lipofectin-treated control cells by 72 ± 7% (mean ± SE; P < 0.001), 70 ± 9% (P < 0.04), and 56 ± 10% (P < 0.02), respectively (data are percentage ranges of three independent experiments). In contrast, UC treatment did not significantly alter Mcl-1 levels in any of the cell lines [SW872, −10 ± 7%; HT1080, 0 ± 20%; SW1353, −13 ± 6% (mean ± SE)]. We also observed similar effects at higher oligonucleotide concentrations in the absence of uptake-enhancing lipids (data not shown). Notably, treatment of sarcoma cells with Mcl-1 ASOs did not change Bcl-2 or Bcl-xL protein levels in the two sarcoma cell lines evaluated (Fig. 1C). We next studied the down-regulation of Mcl-1 protein in a time-course experiment up to 48 h after oligonucleotide administration (Fig. 1B). We detected a pronounced down-regulation of Mcl-1 protein beginning from 8 h up to 36 h after oligonucleotide treatment. Forty-eight h after oligonucleotide administration, Mcl-1 protein levels where indistinguishable from those of untreated cells.

Mcl-1 ASOs Sensitize SW872 Cells to Staurosporine in Vitro. We next investigated the biological relevance of Mcl-1 as an antiapoptotic protein in human sarcoma. SW872 human liposarcoma cells were transfected with Mcl-1 ASOs, UC, or lipofectin alone for 4 h. After transfection, cells were cultured in complete medium and treated with staurosporine (1, 2, 5, and 10 ng/ml) as an apoptosis inducer (46, 47). Twenty-four h later, we evaluated cell growth by quantifying the amount of cellular DNA. As shown in Fig. 2, treatment with Mcl-1 ASOs plus staurosporine (2 ng/ml) cleared decreased cell numbers to 46 ± 6% (mean ± SE; P < 0.004) of control levels compared with UC/staurosporine-treated cells (84 ± 6%; P < 0.04). Oligonucleotide monotherapy had no significant effect on cell growth.

Mcl-1 Protein Expression Is Reduced by Antisense Treatment in Human Sarcoma Xenotransplants. To test whether Mcl-1 ASOs were also capable of down-regulating Mcl-1 expression in vivo, we administered Mcl-1 ASO, UC, or saline as a vehicle control to SCID mice bearing s.c.-implanted SW872 liposarcoma xenotransplants. After 14 days of treatment, the Mcl-1 expression levels in the human sarcoma xenografts were evaluated by immunohistochemistry (Fig. 3).
Mcl-1-specific staining was found in all specimens evaluated; however, Mcl-1 levels were markedly reduced in ASO-treated sarcomas compared with mice that received either saline or UC.

Mcl-1 ASOs Treatment Chemosensitizes Human Sarcoma in Vivo. On the basis of these findings, we tested whether reduction of Mcl-1 expression has the potential to enhance chemosensitivity of SW872 liposarcoma xenotransplants. Tumor size measurements during the course of the experiment revealed significantly reduced tumor growth in mice treated with Mcl-1/CPA compared with all other treatment regimes (Fig. 4A). We confirmed this observation by measuring tumor weight at the end of the experiment (Fig. 4B). The combination of Mcl-1 ASOs and low-dose CPA resulted in a significantly lower mean tumor weight (mean ± SE, 0.44 ± 0.2 g) compared with saline plus CPA (1.19 ± 0.2 g; *P* < 0.01) or UC plus CPA (1.26 ± 0.1 g; *P* < 0.006). When we evaluated the influence of oligonucleotide monotreatment, we found no significant differences in the mean tumor weights of mice treated with ASOs (mean ± SE, 1.39 ± 0.2 g) or UC alone (1.20 ± 0.2 g) compared with those treated with saline (1.38 ± 0.3 g).

Assessment of Apoptosis in Human Sarcoma Xenografts. To assess whether the chemosensitization effect of the Mcl-1 ASOs was associated with a higher rate of apoptotic cell death in tumor xenografts, we examined tumors for cell death by TUNEL staining (Fig. 5A). Treatment with Mcl-1 ASOs plus CPA increased the number of apoptotic cells ~3-fold (11% apoptotic cells) in SW872 sarcoma xenotransplants compared with the saline plus CPA or the UC plus CPA control group (3 and 3% apoptotic cells, respectively). A decisive point during apoptosis is the proteolytic activation of caspase 3 from its proform. We therefore analyzed tumor sections immunohisto-
Mcl-1 ASOs previously shown to be capable of down-regulating Mcl-1 protein via the use of Mcl-1 ASOs. In our study, we used oligonucleotides (ASOs) to target Mcl-1 expression and lowered the apoptotic threshold in neoplastic cells (51-53), we addressed the importance of Mcl-1 expression and lowered the apoptotic threshold in human sarcoma cells and xenotransplants by down-regulating Mcl-1 protein via the use of Mcl-1 ASOs. In our study, we used Mcl-1 ASOs previously shown to be capable of down-regulating Mcl-1 expression in endothelial (54) and melanoma cells (42, 55). In vitro experiments demonstrated a strong, rapid, and specific down-regulation of the Mcl-1 target protein in all three sarcoma cell lines tested. The antisense effect was already observed after 8 h and lasted for 36 h. More importantly, we could also demonstrate a strong Mcl-1 down-regulation in human liposarcomas grown in SCID mice after systemic administration of the Mcl-1 ASOs.

Notably, it is rather unlikely that immunostimulation by the Mcl-1 ASOs might be responsible for the antitumor effects observed. First, because the Mcl-1 ASOs used in the study are human specific, with a five-base mismatch to the murine Mcl-1 mRNA sequence, it was mandatory to study the Mcl-1 ASOs in a xenograft model using immunocompromised SCID mice lacking mature B and T cells. Even if the use of such a model does not exclude any immunostimulation by itself, a major antitumor response caused by a putative immunostimulation seems not to be very likely. Second, the Mcl-1 ASOs used in this study did not contain any immunostimulatory CpG motif or any other published immunostimulatory sequence motifs. Furthermore, gross pathological examination did not reveal splenomegaly in any of the Mcl-1 ASOs- or UC-treated mice. Splenomegaly has been reported in a variety of SCID mice models after administration of oligonucleotides with immunostimulatory potential. Therefore, both of these points further substantiate the notion that the observed effects are at least predominantly due to the chemosensitization potential of the Mcl-1 ASOs used.

The observation that expression of Bcl-2 and Bcl-xL, two other antiapoptotic members of the Bcl-2 family with homology to the Mcl-1 protein (12, 56), was not altered by Mcl-1 ASOs treatment in vitro supports the notion that the effect of down-regulation of Mcl-1 on tumor growth is not due to accompanying changes in the expression of other Bcl-2 family members. Furthermore, Mcl-1 ASOs with the same sequence as the ones used in our study have recently been reported to down-regulate Mcl-1 in endothelial cells; a control oligonucleotide that contains four mismatches had no effect on Mcl-1 expression levels (54).

We showed that Mcl-1 down-regulation by ASOs has the potential to enhance chemosensitivity in human sarcoma in vitro and in vivo. Treatment of sarcoma cells in vitro with a Mcl-1 ASOs followed by subsequent application of the experimental apoptosis inducer staurosporine resulted in increased tumor cell death compared with UC/staurosporine- or saline/staurosporine-treated cells. In view of these in vitro experiments, we evaluated our concept in a SCID mouse xenotransplantation model. Combined treatment with Mcl-1 ASOs plus CPA resulted in clearly enhanced tumor cell apoptosis and led to a significantly reduced mean tumor weight and tumor volume compared with UC/CPA- or saline/CPA-treated animals. Notably, neither Mcl-1 ASOs nor CPA single-agent treatments resulted in major antitumor activity.

CPA, an alkylating agent commonly used in antineoplastic therapy, is an isomeric cancer prodrug that is bioactivated by specific cytochrome P-450 enzymes to form therapeutically active, cytotoxic metabolites (44, 45). The rationale of our work was to use ASOs directed against the antiapoptotic protein Mcl-1 to sensitize human sarcoma to low-dose CPA chemotherapy. This approach carries not only the potential to increase the response rate of treatment-resistant human sarcoma to CPA, but also minimizes the toxic side effects of CPA observed at standard...
ard doses. Furthermore, the administration of low-dose CPA has been reported to enhance the immune response both in experimental animals (57, 58) and in humans (59, 60), leading to tumor rejection.

In conclusion, our data highlight the role of Mcl-1 as a chemoresistance factor in human sarcoma and suggest that the combination of Mcl-1 ASOs with low-dose CPA may qualify as a highly effective and promising treatment strategy in human sarcoma.

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