Increase of Cisplatin Sensitivity by c-myc Antisense Oligodeoxynucleotides in a Human Metastatic Melanoma Inherently Resistant to Cisplatin

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ABSTRACT

In this study, we evaluated the role of the c-myc oncogene in response to cisplatin (DDP) treatment using two melanoma lines derived from the primary tumor (LP) and metastatic lymph node (LM) of the same patient. These cell lines, which retain the phenotypic profile of the original tumors, were studied for growth behavior, expression of c-Myc oncoprotein, and HLA-I antigen. The LM line shows a higher tumorigenic ability, an increased expression of c-Myc protein, and a lack of HLA-I antigen, compared with the LP line. In addition, LP tumor was relatively sensitive to DDP administration, whereas LM tumor was resistant to DDP treatment.

To verify whether the increased c-Myc expression observed in the LM line might be responsible for DDP resistance, a c-myc antisense phosphorothioate oligodeoxynucleotide ([S]ODN) was used to down-regulate c-Myc expression. The administration of DDP plus c-myc antisense [S]ODNs produced a decrease in c-Myc protein levels of ~50%, accompanied by a tumor weight inhibition of 65%, similar to that obtained when the sensitive line was treated with DDP alone (tumor weight inhibition = 70%).

Analysis of apoptosis demonstrated that the sensitivity to DDP of the LP line was related to the ability of tumor cells to undergo apoptosis. Conversely, DDP treatment was not able to induce apoptosis in the LM line, whereas apoptosis was evident both after treatment with c-myc antisense [S]ODNs alone and, more extensively, in combination with DDP.

INTRODUCTION

Incidence and mortality of human melanoma has risen rapidly in recent years (1–3). Melanoma is frequently accompanied by alterations in several oncosuppressor genes and oncogenes (4–6). Among these, the c-myc proto-oncogene seems to play an important role in melanoma progression. Recently, investigators have reported that the expression of c-Myc oncoprotein represents a new prognostic marker in cutaneous melanoma and that high expression of c-Myc could predict outcome in both primary and metastatic disease (7, 8). It has also been demonstrated that the expression of c-myc is associated with Clark’s level (9). The role of the c-myc proto-oncogene in the response to antineoplastic drugs is controversial. In fact, in some tumors, overexpression of c-Myc increases sensitivity to several antineoplastic drugs by activating the apoptotic program (10, 11). In contrast, in other systems, the activation of c-Myc contributes to chemoresistance to DDP3 and other drugs (12–14).

We have previously demonstrated that c-myc antisense [S]ODN is an effective inhibitor of human melanoma cell growth in vitro and in vivo (15) and that c-myc antisense [S]ODN treatment enhances the antitumoral efficacy of DDP in a human melanoma model that is relatively sensitive to DDP (16), indicating that the combination of antisense [S]ODN and conventional drugs represents a promising approach in melanoma treatment.

However, research in this field is hampered by the lack of models that mimic the human situation, in terms of either aberrant expression of oncogenes or drug sensitivity. The majority of models consist of transfected cells, and generally, drug resistance is induced by treatment with chemotherapeutic drugs at low doses for long periods.

In an attempt to mimic the human situation as closely as possible, a human melanoma experimental model was used. Two cell lines were derived from the primary tumor (LP) and the metastatic supraclavicular lymph node (LM) of the same patient. In vitro and in vivo growth characteristics as well as expression of c-Myc oncoprotein and HLA-I antigen, shown to

Received 5/3/99; revised 6/24/99; accepted 6/29/99.

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1 This work was supported in part by grants from the Italian Association for Cancer Research, Italy–United States Program and the Ministero della Sanità.

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3 The abbreviations used are: DDP, cisplatin; [S]ODN, phosphorothioate oligodeoxynucleotide; TWI, tumor weight inhibition; IHC, immunohistochemistry; mAb, monoclonal antibody; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
be correlated with melanoma progression, were assessed. Furthermore, the sensitivity to DDP, a drug used in clinical trials for melanoma, was evaluated on the two lines. The line originated from the metastasis showed increased expression of c-Myc protein and decreased sensitivity to DDP, compared with the line derived from the primary tumor. Therefore, the metastatic line was treated with a combination of DDP and c-myc antisense [S]ODNs. The results obtained demonstrate that the administration of c-myc antisense [S]ODNs following DDP treatment potentiates the drug’s effect by activating the apoptotic program.

MATERIALS AND METHODS

Tumor Cell Lines and Tumor Implants. In this study, two human melanoma cell lines derived from a nodular primary cutaneous melanoma (LP; Clark’s level V, Breslow 12 mm) and a supraclavicular metastatic lymph node (LM) of the same patient, who had not received therapy before surgical treatment at the I Surgery Department of the Regina Elena Cancer Institute (Rome, Italy), were used. After mechanical disaggregation of the biopsy fragments, tumor lines were maintained in vitro for 20 passages as monolayer cultures in RPMI supplemented with FCS (10%), penicillin (100 µg/ml), streptomycin (100 µg/ml), and l-glutamine (2 mM) at 37°C in a 5% CO₂–95% air atmosphere in a humidified incubator. The two cell lines were also inoculated into nude mice by injection of 10⁶ cells/mouse into the hind leg muscle of mice. All in vivo experiments were carried out at the second passage of the tumors in nude mice. LP and LM were transplanted into nude mice after mechanical disaggregation of tumors, and tumor cell viability was 70–80%, as determined by trypan blue dye exclusion test.

Drug and [S]ODNs. Clinical-grade DDP (Pronto Platamime, Pharmacia Milan, Italy) was freshly prepared before each experiment and diluted in Earle’s medium to the desired final concentrations. A 15-mer antisense [S]ODN (5'-AAAGT-CATAGGGGCTAGG-3') that was complementary to the translation initiation region of c-myc mRNA and the control scrambled sequence [S]ODN, containing the “G-quartet” motif (5'-AAGTGAGGGGTGT-3'), were obtained from INEX Pharmaceuticals (Vancouver, British Columbia, Canada) and used for all of the experiments (16).

In Vitro Cell Growth. The growth of LP and LM cell lines was assessed by seeding 5 × 10⁴ cells in 60-mm Petri dishes (Nunc; Maccia Brunelli, Milan, Italy). Cell counts (Coulter counter, model ZM; Kontron, Luton, England) and viability (trypan blue dye exclusion) were determined daily, from day 1 to 11 of culture. The doubling time of the cell population was calculated from the growth curves during the exponential phase of the growth. To evaluate cell colony-forming ability, we seeded aliquots of cell suspension of each line into 60-mm Petri dishes with complete medium and incubated at 37°C in a 5% CO₂–95% air atmosphere. After 10–12 days, colonies were stained with 2% methylene blue in 95% ethanol and counted (≥50 cells equaled one colony).

In Vivo Experiments. Male CD-1 nude (nu/nu) mice (6–8 weeks old and 22–24 g in body weight) were purchased from Charles River Laboratories (Calco, Italy). All procedures involving animals and their care were described previously and were in accordance with institutional guidelines in compliance with national and international laws and policies (16).

To assess in vivo tumorigenicity of LP and LM cells, we injected different numbers of tumor cells (from 10⁶ to 3.2 × 10⁷ cells/mouse; Ref. 17). Mice were observed daily to establish the take and the time of tumor appearance. To evaluate the antitumor efficacy of DDP alone or in combination on LP and LM tumors, we injected mice with 10⁶ cells per mouse. DDP was administered i.p. at 10 mg/kg (10% lethal dose, LD₁₀) for 3 consecutive days (3.3 mg/kg) starting at day 8, when a tumor mass of ~200 mg was evident in all mice. Two cycles of treatment were administered at days 8–10 and 21–23 after tumor implant. In the combination experiment, mice received i.p. DDP at 3.3 mg/kg/day at days 8–10, followed by i.v. c-myc antisense [S]ODNs administration in alternating doses of 1 and 0.5 mg/day at days 9–16. This scheduling was chosen based on previous studies (16). Five days after the end of the first cycle of treatment, a second cycle was administered.

Tumor weight and TWI were calculated as reported previously (16).

IHC. Melanoma differentiation antigens gp100 and MelanA/Mart-1 were immunocytochemically evaluated on cytospins obtained from LP and LM melanoma cultured cells and on 3-μm sections cut from ex vivo and in vivo formalin-fixed and paraffin-embedded LP and LM lesions. Cytospins, fixed 10 min in cold acetone, and paraffin sections, mounted on 3-amino propylthiethoxylane (Sigma, Milan, Italy), were stained using the mAb HMB45 specific for gp100 protein (DAKO, Milan, Italy) and the mAb A103 directed to human MelanA/MART-1 (Biogenex; Menarini, Florence, Italy).

HLA-I expression on the tumor cells was determined on frozen sections, fixed 10 min in cold acetone, by sharing with the mouse mAb W6.32 against monomorphic HLA-I determinant (DAKO), whereas c-Myc expression was analyzed on paraffin sections stained with the mAb 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA). Frozen and paraffin sections were processed with a three-step streptavidin biotin immunoperoxidase staining system (LSAB kit; DAKO). The enzymatic activity was developed using 3-amino-9-ethylcarbazole (DAKO) as chromogenic substrate. Following counterstaining with Mayer’s hematoxylin, sections were mounted in aqueous mounting medium (Glycergel; DAKO). Negative controls consisted of parallel sections in which the primary antibody was omitted. Slides were observed by two independent observers. For HLA-I staining, vessels and tumor stroma served as an internal positive control.

Western Blot Analysis. To evaluate the expression level of c-Myc in cultured cell lines or in solid tumors, we solubilized 1 × 10⁶ cells or 100 mg of mechanically disaggregated tumor in lysis buffer. Each tumor lysate was obtained from a pool of three different tumors. Western blot and detection were performed as reported previously (16).

TUNEL Assay. The immunohistochemical detection of apoptosis in formalin-fixed, paraffin-embedded tumor tissues was performed by TUNEL assay (In situ cell death detection kit, fluorescein; Boehringer Mannheim, Milan, Italy). Following deparaffinization and rehydration, 50 µl of TUNEL reaction mixture were applied to the sections, and the slides were incu-
bated at 37°C for 60 min. The slides were then washed three times in PBS for 5 min, and the sections were examined by fluorescence microscope. For each tumor, three different sections were used.

**Statistical Analysis.** Results were analyzed by the Mann-Whitney nonparametric test. Differences were considered significant at $P < 0.05$ (two-sided).

**RESULTS**

**In Vitro Growth and Tumorigenicity of LP and LM Lines.** To obtain a preclinical model that was suitable for investigating drug sensitivity during the life history of tumors, we used two lines that originated from a primary tumor and a metastatic lesion of the same patient. As summarized in Table 1, LP and LM cultured cell lines and the correspondent xenografts maintained the same antigenic profile of the ex vivo primary and metastatic melanoma. In fact, IHC that was performed to compare ex vivo, in vitro, and in vivo expression of melanoma differentiation antigens gp100 and MelanA/MART-1 in LP and LM lesions demonstrated an overlapping expression of the two antigens.

Table 2 shows the in vitro and in vivo growth behavior of the LP and LM lines. The two lines show different in vitro growth in terms of doubling time, saturation density, and clonogenicity. It is also evident that the LM line exhibited a higher tumorigenic ability than the LP line in terms of both tumor take and median time of tumor appearance.

Because melanoma progression is frequently accompanied by overexpression of c-myc oncogene, associated with HLA-I down-regulation, we analyzed HLA-I and c-Myc protein expression in ex vivo and in vivo LP and LM lesions by IHC. Table 2 shows that the expression of HLA-I in ex vivo (Fig. 1a) and in vivo (Fig. 1b) primary melanoma was comparable, whereas the corresponding metastatic lesion showed a marked down-regulation of HLA-I molecule (Fig. 1, Lanes 1 and 2). In contrast, the expression of c-Myc protein, normalized to $\beta$-actin levels, both in vitro and in vivo primary melanoma (Fig. 1, Lanes 3 and 4) was comparable, whereas the corresponding metastatic lesion showed a marked down-regulation of c-Myc protein expression (Fig. 1, Lanes 5 and 6). The in vitro LP and LM lines showed HLA-I and c-Myc expression that was similar to that observed in in vivo and ex vivo tumors.

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Fig. 1. a–d, expression of HLA-I product between primary (LP) and metastatic (LM) lesions, as revealed by streptavidin-biotin immunoperoxidase staining. Frozen sections of LP are homogeneously positive for HLA-I in both ex vivo (a) and in vivo specimens (b), whereas loss of HLA-I is shown in the LM lesions (c and d). In ex vivo LP, normal skin (a, arrowhead) serves as an internal positive control. Original magnifications, ×250 (a) and ×400 (b–d). e–f, immunostaining of c-Myc protein in LP and LM tumors. LP shows a weak and heterogeneous IHC staining for c-Myc oncogene product in both ex vivo (e) and in vivo (f) tumors. In contrast, LM displays a strong nuclear and cytoplasmic immunostaining for the oncoprotein (g and h, ex vivo and in vivo, respectively). In the LM lesion (g), normal lymphocytes (arrowhead) are completely negative. Original magnifications, ×250 (e) and ×400 (f–h).
Antitumor Efficacy of DDP Alone or in Combination with c-myc [S]ODNs. The efficacy of DDP was evaluated in mice bearing the LM and LP tumors. DDP was administered according to two repeated cycles, on the basis of previous results demonstrating that repeated administrations were more effective than a single bolus (16). DDP treatment for 3 consecutive days started at day 8 after cell implantation, when the tumor mass was ~200 mg. The interval time of 10 days between the first and the second cycle of treatment was chosen on the basis of complete recovery from the body weight loss observed after the first cycle. Fig. 3 shows the tumor growth curves of treated and untreated animals implanted with LP (A) and LM (B) cells. It is evident that two cycles of DDP treatment induces a significant inhibition of LP tumor volume of ~70% (at the second cycle of treatment; Fig. 3A). On the contrary, the reduction in LM tumor mass was similar after the first and the second cycle of treatment (TWI of ~30% at the nadir; Fig. 3B).

On the basis of results demonstrating that the LM line shows high levels of c-Myc protein and, in parallel, a high resistance to DDP and considering that c-myc oncogene is involved in DDP resistance (12, 14), we verified whether the modulation of c-Myc expression might increase DDP efficacy on LM line. Mice were treated with DDP followed by the administration of c-myc antisense [S]ODNs. Fig. 3B shows that single-agent treatment with c-myc antisense [S]ODNs induced a higher reduction of tumor mass (TWI at the nadir was ~50%) than DDP treatment. In addition, DDP efficacy was increased when c-myc antisense treatment followed DDP administration. In fact, this combination produced decreases in tumor mass of ~50% after the first cycle of treatment and ~65% after the second cycle. These results clearly demonstrate that c-myc antisense [S]ODNs treatment was able to increase DDP efficacy. The values of tumor inhibition obtained using this combination are similar to those obtained treating the relatively sensitive LP tumors with DDP. The effects of c-myc scrambled [S]ODNs, alone and in combination with DDP, on tumor growth were superimposable on the effects observed in untreated and DDP-treated tumors, respectively (data not shown).

To ascertain whether the c-myc antisense [S]ODNs effect was due to a down-regulation of c-Myc protein, we performed Western blot on tumor lysates that were excised 3 days after the end of the two treatments (Fig. 4). Immunoblot analysis demonstrated that c-Myc protein levels were reduced by ~50% after treatment with c-myc antisense [S]ODNs, alone or in combination with DDP. Conversely, the c-Myc protein expression in DDP-treated tumors was increased to ~25–30%, compared with untreated tumors. c-Myc protein expression after treatment with c-myc scrambled [S]ODNs, alone and in combination with DDP, was similar to the expression observed in untreated and DDP-treated tumors, respectively (data not shown).

To clarify the mechanism(s) by which LP and LM lines show differences in chemosensitivity to DDP, we analyzed the ability of different in vivo treatments to trigger apoptosis in the...
We previously demonstrated that both DDP treatment and down-regulation of c-Myc protein exert their action through induction of apoptosis (15, 16). Fig. 5 shows TUNEL staining in tumor sections of LP and LM cells after different treatments. Treatment with DDP was able to induce apoptosis in the LP tumor but not in the LM tumor. In fact, apoptotic cells and nuclear DNA fragmentation were clearly evident after treatment with DDP in LP tumor sections (Fig. 5b) but not in tumor sections of LM cells (Fig. 5d). Moreover, apoptotic cells were observed in LM tumor exposed to c-myc antisense [S]ODNs alone (Fig. 5e), but the apoptotic cell death was more extensive in the tumors treated with the combination (Fig. 5f). The data presented here suggest that c-myc oncogene contribute to the lack of chemosensitivity of human melanoma that can be overcome by c-myc antisense therapy.

DISCUSSION

Resistance to chemotherapeutic agents represents one of the major obstacles to the success of cancer therapy (18, 19). The majority of drug resistance studies are performed on in vitro and in vivo models originating from cell lines selected in chemotherapeutic drugs. These artificial systems show elevated levels of drug resistance and do not necessary mimic the clinical
tumor resistance. In fact, the chemoresistance that arises during the progression of disease exhibits a rate lower than that observed on in vitro lines. Therefore, the availability of experimental models that mimic the clinical situation could be useful for the evaluation of the potential of combination therapies.

To this purpose, we used two melanoma cell lines originating from a primary (LP) and a metastatic (LM) lesion of the same patient. The IHC expression of the two melanoma differentiation antigens, gp100 and MelanA/Mart 1, clearly demonstrates that the two lines retain the same antigenic profile of the original tumors. Moreover, the LM line showed higher levels of c-Myc protein than did the LP line. These results are in agreement with other studies demonstrating that c-Myc overexpression correlates with melanoma progression and invasion (8, 9, 20). In fact, it has been observed that, in melanoma lesions, c-Myc protein overexpression predicts poor outcome and is associated with reduced disease-free interval (7). In addition, LM tumors show a marked down-regulation of the HLA-I molecule compared with LP tumors both in ex vivo and in vivo lesions. This is in accordance with other studies reporting that c-Myc overexpression is accompanied by a decrease of HLA-I protein, suggesting that reduction in the amount of class HLA-I molecule is associated with increased malignancy and that c-Myc overexpression may influence the immune response in melanoma (8, 21, 22).

The phenotypic differences observed in the LP and LM melanomas correlate with the variation in biological parameters observed both in vitro and in vivo. In particular, LM cells displayed acquired biological advantages both in terms of cell proliferation and clonogenic ability. These results were consistent with the increased in vivo tumorigenic ability elicited by LM tumor, as is evident from the different tumor takes and the median time of tumor appearance. Taken together, our results indicate that the LP and LM lines mimic human melanoma progression, allowing us to study the efficacy of antineoplastic treatments.

The effects of DDP were different in the two lines. A marked tumor growth inhibition (70%) was observed treating the mice bearing the LP tumors with two cycles of DDP administration, whereas only a slight reduction in tumor mass (~30%) was obtained using the same DDP dose and schedule in mice bearing LM tumors.

Although the molecular basis of DDP resistance is poorly understood, several oncogenes have been implicated in this resistance as well as c-myc (12, 14, 19). Nevertheless, the role of c-myc in drug resistance is still controversial. Some studies have shown that increased c-myc expression enhances sensitivity to anticancer drugs (10, 11), whereas other studies demonstrate that elevated c-Myc expression determines drug resistance (12–14). Recently, it was demonstrated that down-regulation of endogenous c-myc expression, using an antisense c-myc gene transfer, increased DDP sensitivity but not doxorubicin or vincristine sensitivity of a drug-resistant human small cell lung carcinoma line (23). The different responses to DDP and the different expression of c-Myc elicited by LM and LP tumors suggest that c-myc can play an important role in DDP resistance of melanoma. The relevance of c-myc in DDP responsiveness also appears clear from the demonstration that specific targeting of c-myc oncogene by antisense [S]ODNs increase DDP antitumor efficacy in LM melanoma cells. In fact, DDP treatment followed by c-myc antisense [S]ODNs reduces the LM tumors mass to a value similar to LP tumors treated with DDP alone. This is in agreement with several in vitro studies, which suggested that elevated c-Myc expression can confer resistance to DDP (12–14). The increased sensitivity to DDP in LM tumor is specifically due to an antisense mechanism because a down-regulation of c-Myc protein has been observed. Conversely, DDP treatment induced an increase in c-Myc protein levels. The ability of DDP treatment to increase c-Myc expression after DDP treatment has also been reported by other authors in in vitro experiments as well as in freshly isolated colon carcinoma tissue from patients with failed DDP therapy (24). It has been hypothesized that a specific induction of c-Myc protein expression after DDP treatment could be due to the presence of a DDP responsive element within the human c-myc gene promoter (25, 26).

The different effects on tumor growth inhibition observed in LP and LM tumors after DDP treatment could be related to the inability of LM cells to undergo apoptosis. In fact, only tumors originating from the primary tumor showed apoptotic cells after DDP treatment. Conversely, DDP treatment was not able to induce apoptosis in the LM line, whereas apoptosis was evident after treatment with c-myc antisense [S]ODNs, both alone and, more extensively, in combination with DDP.

The mechanism by which c-myc antisense increases the DDP sensitivity could be due to the induction of apoptosis as a consequence of cell cycle perturbation induced by the two agents. In fact, as we have already demonstrated, the treatment with c-myc antisense [S]ODNs prevents recovery from G2–M phase accumulation induced by DDP activating the apoptotic program (16). These data suggest that c-Myc overexpression contributes to DDP resistance of human melanoma and that the induction of apoptosis by c-myc antisense [S]ODNs increases the DDP sensitivity. In addition, we have also demonstrated that the DDP-[S]ODN combination caused a marked reduction of bcl-2 expression. We think that the improved antitumoral efficacy of DDP-[S]ODN treatment could be due to different cooperating mechanisms, including a direct interaction between DDP and c-Myc, an activation of the apoptotic program induced by antisense per se, and an inhibition of recovery of the damage induced by DDP.

Our findings demonstrating the ability of c-myc antisense [S]ODNs to increase in vivo sensitivity of human metastatic melanoma may be of considerable relevance. Thus, the use of antisense [S]ODNs is a promising approach for altering c-myc gene expression or c-Myc function, and it may be important in the quest to overcome drug resistance in melanoma patients, whose tumors carry an overexpressed c-myc gene.

ACKNOWLEDGMENTS

We thank Inex Pharmaceuticals for kindly providing c-myc oligodeoxynucleotides. We are grateful to Sara Moretti for expert technical assistance and to Simona Righi for excellent secretarial assistance in preparation of this manuscript. We also thank Paula Franke for formal language revision of this manuscript.
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*Clin Cancer Res* 1999;5:2588-2595.

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