Suppression of Dexamethasone-induced Metallothionein Expression and cis-Diamminedichloroplatinum(II) Resistance by v-mos

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ABSTRACT

Metallothionein has been implicated in resistance to anticancer drugs. We examined whether transient induction of metallothionein by dexamethasone causes resistance to cis-diamminedichloroplatinum(II) (cis-DDP) in malignant and nonmalignant cells. Normal rat kidney cells (6m2) were infected with a modified v-mos oncopGene construct in which expression of v-mos and consequently transformation was temperature-sensitive occurring at the permissive temperature of <33°C and not at the nonpermissive temperature of 37°C. Temperature-sensitive oncogenic transformation by v-mos attenuated induction of metallothionein by dexamethasone. No induction of metallothionein was observed in a revertant 6m2 cell line (54-S4A), which expressed v-mos and was transformed at 37°C. Only nontransformed 6m2 cells displayed resistance to cis-DDP after dexamethasone pretreatment for 24 h. Dexamethasone pretreatment did not cause marked resistance to doxorubicin or melphalan in nontransformed 6m2 cells. When 6m2 cells (37°C) were pretreated with dexamethasone (0.5 μM) for 24 h and then incubated in dexamethasone-free medium for 24 h, both metallothionein levels and resistance to cis-DDP decreased significantly. Thus, transient resistance to cis-DDP can be produced by a nonmetal inducer of metallothionein in nontransformed cells. Glucocorticoid-induced protection is suppressed in cells expressing v-mos and this might form the basis of future strategies to improve the therapeutic index of cis-DDP.

INTRODUCTION

MTs are the major intracellular protein thiols in eukaryotes (1, 2). They are a family of highly conserved low molecular weight proteins that have been observed in the cytosol and nucleus of both normal and malignant cells (3, 4). The high cysteine content of MTs permit them to bind a wide variety of heavy metals such as Zn2+, Cu2+, Hg2+ or Cd2+ and they are clearly involved in heavy metal detoxification and in metal homeostasis (1, 2).

MTs can be induced by a host of environmental and therapeutic substances. Indeed, cis-regulatory elements have been identified for many substances in the 5′-flanking region of MT genes including a well described group of glucocorticoid-regulatory elements (1, 2). The elaborate variety of endogenous cis-regulatory devices for MT transcription has led to the hypothesis that MTs have other unidentified functions (1, 5). One potential function may be to protect cells against substances that covalently interact with DNA (5–8). Cells that are chronically resistant to the DNA-reactive agents, such as cis-DDP, often overexpress MT (7). Cells chronically resistant to Cd2+ that overexpress MT are always cross-resistant to cis-DDP (6, 7, 9). Finally, cells transfected with bovine papilloma virus containing human MT-IIA are resistant to alkylating agents, cis-DDP, and mutagens (7, 8). Despite this evidence, some controversy remains concerning the role of MT in drug resistance (10).

The lack of cytotoxic specificity to malignant tissues remains a major limitation with current chemotherapy. It is now evident that expression of oncoproteins is a central hallmark of malignancy. These proteins often interfere with normal signal transduction pathways. For example, transformation with H-ras and other oncogenes results in alterations of inositol phospholipid metabolism (11). The cellular expression of p21 H-ras, p37 v-mos, or p60 v-src produces a strong repression of glucocorticoid-dependent promoters and promoter constructs (12). In particular, v-mos expression has been shown to suppress the ability of glucocorticoids to differentiate bone marrow preadipocytes (13) and to increase mRNA encoding for rat MT-I (14). There have, however, been few successful attempts to capitalize therapeutically on the known biochemical actions of oncogenes. Thus, we have examined the role of v-mos expression on the inducibility by dexamethasone of MT protein and on cellular resistance to cis-DDP. We have chosen dexamethasone because it is widely used to suppress the nausea and vomiting associated with cis-DDP therapy, it has a role in the palliative management of some malignancies, and it is a strong inducer of MT (14). Normal rat kidney cells have been used because they are derived from the organ that is most sensitive to the toxic effects of cis-DDP. These cells can be infected with a modified v-mos construct in which expression of v-mos and consequently transformation is temperature sensitive allowing for evaluation of the effects of dexamethasone, which are strictly limited to changes in transcription.

MATERIALS AND METHODS

Materials. MT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dexamethasone, and melphalan were purchased from Sigma Chemical Co., St. Louis, MO. cis-DDP was obtained from Bristol-Myers Squibb Co., Wallingford, CT, and doxorubicin was from Adria Laboratories, Inc., Columbus, OH. DACH was generously provided by Dr. Miles P. Hacker, Department of Pharmacology, University of Vermont, Burlington, VT.

Cell Culture. Normal rat kidney cells infected with a temperature-sensitive Moloney murine sarcoma virus ts110 (6m2) were kindly provided by Dr. Donald B. DeFranco (Department of Biological Sciences, University of Pittsburgh). Below 33°C (permissive temperature), 6m2 cells are transformed and express an 85 kDa gag-mos gene fusion protein (p85mos−), which contains a normal mos-like serine/threonine protein kinase activity (14). The production of this p85mos− fusion protein is strictly dependent on a temperature-sensitive splicing event that produces a 3.5-kilobase mRNA. At the nonpermissive temperature (37°C), an in-frame translational stop codon is functional in the unspliced viral mRNA (4 kilobases); the resultant translation product is a...
RESULTS

Induction of MT by Dexamethasone in Transformed and Nontransformed 6m2 Cells. 6m2 cells grown at 31°C express v-mos and are phenotypically transformed (14). Because oncogenic transformation by v-mos is known to alter glucocorticoid-in- sensitive temperature. These cells (54-5A4) have a 5-base pair deletion within the Moloney murine sarcoma virus provirus that eliminates a splicing site as well as the premature translational termination codon making them a useful control cell population for comparisons with the 6m2 cells (15). These cells also were kindly provided by D. B. DeFranco. All cells were maintained as monolayer cultures in MeCoy's 5A medium (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 15% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/ml), and streptomycin (100 units/ml) and kept in a humidified incubator at 37°C or 31°C in 5% CO2-95% air. Dexamethasone dissolved in ethanol was diluted in the growth medium and was added to the plate as described in the text. The concentration of ethanol never exceeded 0.1% and ethanol was always added to the control cultures. Unless otherwise mentioned, cells were preincubated with dexamethasone for 24 h and then incubated with a single exposure to various concentrations of anticancer drugs. Following a 3- to 72-h exposure to the anticancer drug, the number of viable cells was determined by enzyme-linked immunosorbent assay as described in the text. Each mean value was obtained from 4 or more determinations made for each mean value obtained in a single experiment. Bars, SE.

Assessment of MT Content. The MT content was estimated by an indirect competitive enzyme-linked immunosorbent assay using polyclonal rabbit antibody to rat MT-I as described previously (7).

**Effect of Dexamethasone on the Antiproliferative Activity of cis-DDP.** Since overexpression of MT due to chronic exposure of cells to cis-DDP or cadmium confers resistance to cis-DDP, we examined whether or not acute induction of MT by dexamethasone also caused resistance to cis-DDP. As shown in Fig. 2A, a 24-h pretreatment with 0.5 μM dexamethasone shifted the growth inhibition curve of nontransformed 6m2 cells to the right, increasing the IC50 for cis-DDP 3.3-fold. In contrast, a similar pretreatment with dexamethasone had no effect on the antiproliferative activity of cis-DDP in transformed 6m2 (Fig. 2B) or 54-5A4 cells (Fig. 2C). It is also interesting to note that the relative sensitivity of the untreated transformed 6m2 and nontransformed 6m2 cells was similar. Pretreatment of nontransformed 6m2 cells for 24 h with a dexamethasone concentration as low as 0.05 μM produces a marked increase in the IC50 for cis-DDP (data not shown).

We also studied the effects of dexamethasone on the antiproliferative activity of several other anticancer agents, such as doxorubicin, melphalan, and DACH. A 24-h pretreatment with 1 μM dexamethasone had little effect on the sensitivity of nontransformed 6m2 cells to doxorubicin and DACH and increased their sensitivity to melphalan only slightly (data not shown).

**Effect of Withdrawal of Dexamethasone on cis-DDP Sensitivity and MT Content.** Fig. 3 illustrates the reversibility of the resistance phenomenon with dexamethasone. When nontransformed 6m2 cells were preincubated with 0.5 μM dexamethasone for 24 h and then incubated in dexamethasone-free medium for 4 h before continuous exposure to cis-DDP, the dexamethasone-induced resistance to cis-DDP was reversed. To examine if reversal of cis-DDP resistance was associated with reduction in MT, we determined the MT content in 6m2 cells 24 h after withdrawal of dexamethasone. As shown in Fig. 4, MT levels dropped significantly in cells when dexamethasone was removed (Fig. 1). The difference in temperatures at which nontransformed and transformed 6m2 cells were grown could not account for the MT induction by dexamethasone because dexamethasone failed to induce MT in revertant 54-5A4 cells, which expressed v-mos and exhibited the transformed phenotype when grown at 37°C. These cells had constitutive levels of 6.1 ng MT/mg protein and after pretreatment for 24 h with 0.5 μM dexamethasone they had only 19.2 ng MT/mg protein.

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Fig. 3. Effect of withdrawal of dexamethasone on the antiproliferative activity of cis-DDP. Nontransformed 6m2 cells were pretreated with ethanol vehicle (C) or 0.5 μM dexamethasone (◇, ●) for 24 h and then incubated in either medium containing dexamethasone (◇) or vehicle (●) for an additional 4 h. The antiproliferative activity of continuous cis-DDP exposure was determined after 72 h by a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described in “Materials and Methods.” Each mean value was obtained from 4 or more determinations and these results are representative for 2 separate experiments.

Fig. 4. Effect of withdrawal of dexamethasone on MT and the antiproliferative activity of cis-DDP. Nontransformed 6m2 cells were pretreated with 1 μM dexamethasone for 24 h. Either medium was left for an additional 24 h (unwashed) or the medium was removed. The cells incubated for an additional 24 h in drug-free medium (washed) and the MT levels (◇) were determined. The cellular sensitivity to cis-DDP was measured by continuous exposure to cis-DDP 3 h after cells were washed or not washed. The IC50 (◇) was determined as described in “Materials and Methods.” Bars equal range from 2 experiments.

(1 μM) was removed compared to the cells in which dexamethasone was present continuously in the medium (410% versus 680% of control). Even after a 24-h incubation in dexamethasone-free medium, however, MT levels were considerably higher than the control value. Concomitant to a decrease in MT content, the IC50 for cis-DDP decreased from 390% of control to 170% of control value. (Fig. 4).

DISCUSSION
While it is clear that oncogene products are essential attributes of malignant phenotypes, therapeutic strategies that clearly capitalize on the expression of these oncoproteins are not available. Such an approach certainly seems reasonable.

Glucocorticoids have been widely used in the palliative therapy of lymphomas and leukemias and they may suppress some of the untoward effects of anticancer agents (17). There are, however, no firm mechanistic explanations for these actions. Previous studies indicate that chronic overexpression of MT produces resistance to cis-DDP and alkylating agents, as well as mutagens (7, 8). MT can be induced by a number of therapeutic and environmental agents including heavy metals, cytokines, heat, X-irradiation, and corticosteroids (1, 2). We have examined the effects of pretreatment of normal rat kidney cells in culture with the glucocorticoid, dexamethasone, to determine if an increase in MT by a nonmetal inducer could increase resistance to cis-DDP. Kidney cells are especially interesting because they are derived from an organ that is particularly sensitive to cis-DDP. A 24-h pretreatment with dexamethasone markedly increased cellular MT; this is consistent with previously reported increases in MT mRNA that occur in these cells soon after the addition of dexamethasone (14). This pretreatment with dexamethasone also markedly reduced the antiproliferative effects of a wide range of cis-DDP concentrations. This is a transient effect that can be at least partially reversed after removal of dexamethasone for 24 h. Other investigators have estimated the half-life of MT in cultured cells of 4 to 50 h (18) and this is consistent with our observations. Longer dexamethasone-free periods have not yet been examined because the cell density at the time of cis-DDP treatment with our current protocol would be too great to allow for an accurate evaluation of antiproliferative effects. We hypothesize, however, that MT and cis-DDP sensitivity would return to the levels seen in untreated cells with a sufficiently long dexamethasone-free period.

The resistance to cis-DDP seen after dexamethasone pretreatment was not a nonspecific response because marked resistance to other antineoplastic agents, such as DACH, doxorubicin, or melphalan, was not observed. The failure to detect marked resistance to melphalan was, in fact, surprising because we (7) and others (8) have noted that overexpression of MT leads to resistance to alkylating agents. It is possible, therefore, that dexamethasone pretreatment induced additional cellular factors that could have altered cellular sensitivity to melphalan. The mechanism by which MT may protect cells against cis-DDP toxicity is not fully understood and it could require additional cellular factors. Although it is generally believed that MT can interact with electrophilic antineoplastic agents due to its high nucleophilicity and thereby inactivates them (9), the direct binding of MT to cis-DDP in cells that have not previously been induced by cadmium is yet to be demonstrated. Additionally, the stoichiometry of the potential binding as well as the role of particular isoform(s) of MT and their subcellular localization require further consideration.

The observation of transient resistance to cis-DDP after dexamethasone treatment could be clinically significant. Glucocorticoids are used often in the therapy of leukemias and lymphomas as well as to treat hypercalcemia associated with neoplasia. Patients who are treated with cis-DDP frequently receive dexamethasone to reduce the nausea and vomiting. The antiemetic effects of dexamethasone could be mediated in part by transient elevations in MT in nonmalignant tissues, such as the gastrointestinal tract. This is clearly a testable hypothesis. Furthermore, steroid levels do change in a predictable and circadian manner and induction of MT by these endogenous steroids could be at least a partial basis for the reported diurnal differences in untoward effects seen in patients during chemotherapy with cis-DDP (19). Finally, the transient resistance due to induction of MT by exogenous or endogenous glucocorticoids might add to preexisting stable intrinsic or acquired resistance in malignant tissues that lacked the expression of oncogenes such as v-mos.

Previous studies have described the ability of oncogenes containing serine/threonine protein kinase activity, such as v-mos, to suppress glucocorticoid-induced transcription (12). DeFranco et al. (14, 20) observed that v-mos could suppress
Dexamethasone-induced increases in MT mRNA in 6m2 cells at the permissive temperature and our observed mitigation of induction of MT protein is consistent with this. The inhibition of both MT mRNA and protein induction appears to occur due to a rapid return of the “inactive” glucocorticoid receptor to the cytoplasm (20, 21). The biochemical basis for this inactivation and return to the cytoplasm is not yet well understood. Nevertheless, the presence of this oncogene-dependent suppression of MT induction increases the therapeutic index of cis-DDP at least in this artificial cell culture system. The sensitivity of the glucocorticoid response to at least some oncogene expression may explain why cis-DDP resistance was not seen previously after dexamethasone pretreatment of murine P388, human endometrial, or human colon cancer in mice (22). The oncogene profile of these cells is not known, however, and the pretreatment period was very short. With tumors that contain v-mos or other oncogenes, it may be possible to therapeutically exploit the selective difference in glucocorticoid induction of MT.

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