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Differential Response of Cultured Human Normal and Tumor Cells to Trace Element-induced Resistance to the Alkylating Agent Melphalan[1]

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ABSTRACT

Previous studies using cultured Chinese hamster cells indicated that pretreatment of the cells with the trace elements copper, selenium, and/or zinc resulted in increased survival of the metal-induced cultures following subsequent exposure to mono- and bifunctional alkylating agents. To ascertain whether a comparable protective response could be activated in human-derived material, a series of human normal and tumor cells was treated with these trace elements and later challenged with the alkylating agent melphalan, prior to determination of the surviving fraction via colony formation. Normal human cells derived from either newborn infants or adults exhibited an increase in survival of 7- to 9-fold when pretreated with zinc alone that increased to approximately 16-fold when these normal cells were induced with all three trace elements. In contrast, comparable pretreatment of tumor cell populations resulted in an increase in survival of 1.7-fold or less, with most types of tumors exhibiting no induced protection. These observations describing a differential inducibility of normal and tumor cells raise the possibility of a novel approach for selectively sparing normal tissue in patients undergoing treatment with alkylating agents. Possible ramifications to cancer chemotherapy are discussed.

INTRODUCTION

Alkylating agents are effective antitumor drugs whose major limitation in a clinical setting is a lack of specificity for tumor tissue. That is, actively proliferating normal cells in the hematopoietic system and the gastrointestinal tract are nearly as sensitive to alkylating agents as are tumor cells (7). If the resistance of normal cell populations could be selectively increased in patients undergoing alkylating agent chemotherapy, higher, more effective dose levels could be tolerated.

One possible approach to altering the resistance of cells to alkylating agents was suggested in earlier studies from this laboratory in which the survival of melphalan-treated cultured Chinese hamster cells was increased dramatically when the cells were pretreated with the essential trace elements zinc, copper, and selenium (16–18). While reasons were provided for postulating that the induced protective response might have relevance to the management of cancer in humans (16, 17), the case was not particularly convincing, since: (*a*) the cells utilized were nonhuman and were derived from an aneuploid, tumorigenic cell line (Chinese hamster ovary) (3, 9); and (*b*) no comparisons were made between induced responses of normal and tumor cell populations.

To provide information of greater relevance to the human situation, in this paper, we have examined the ability of zinc, copper, and selenium to induce protection against the alkylating agent melphalan in a series of human-derived normal and tumor cells. The results indicate that a greater degree of resistance is elicited in normal cells than in their neoplastic counterparts, suggesting that the metal induction procedure may, indeed, provide a novel means for selectively sparing normal tissue in chemotherapy regimens using alkylating agents.

MATERIALS AND METHODS

Human Normal Cells. Four normal human fibroblasts were utilized, 2 derived from adult tissue and 2 from newborn tissue. The adult cells were established by Dr. W. Wharton from biopsied chest skin obtained from patients over 35 years of age who were undergoing surgical procedures for the treatment of nonneoplastic diseases (22); these adult fibroblasts were designated UNMA and UNMC. A set of fibroblasts derived from neonate foreskin samples by Dr. D. Chen served as the source of the HSF-7 and HSF-22 newborn cells. The 4 cells were obtained from 4 different individuals. Both adult and newborn cells were grown as monolayers in α-MEM[3] supplemented with 10% FBS. Plating efficiencies in α-MEM:FBS averaged 19% for UNMA, 18% for UNMC, 32% for HSF-7, and 33% for HSF-22. Drug experiments utilized cells from passages 7 to 14 (75% from passages 9 to 11). All cells were determined to be free of infection with *Mycoplasma*.

Human Tumor Cells. A set of human tumor cell lines was selected that encompassed a variety of types of tumors differing in growth properties (*e.g.*, plating efficiencies, culture doubling times, etc.) and resistance to alkylating agents. Two tumor lines were obtained from Dr. J. Fogh, TE-32 rhabdomyosarcoma, also designated RD-2 and 130T (6, 11), and SW-872 liposarcoma (6). HT-1080 fibrosarcoma cells (5, 13) were obtained from Dr. B. Crawford. Four additional tumor lines were provided by Dr. D. Enger: A101D melanoma, also designated Hs294T (6); A549 alveolar cell carcinoma (6, 8, 10); A204 rhabdomyosarcoma (6, 8); and A1663 bladder transitional cell carcinoma (5). All of the cells were determined to be *Mycoplasma* free. Average plating efficiencies in α-MEM:10% FBS were 41% for TE-32, 5% for SW-872, 62% for HT-1080, 25% for A101D, 70% for A549, 50% for A204, and 76% for A1663. Since nontoxic levels of trace elements were chosen for this study, the plating efficiencies of both the normal and tumor cell lines in α-MEM supplemented with trace elements were equivalent to values obtained in α-MEM alone.

Information on therapeutic treatment of the patients from whom the tumor cell lines were derived was obtainable for 3 of the lines. The child from whom TE-32 was obtained received high-dose cyclophosphamide therapy before the biopsy material was obtained (11); thus, these cells might be expected to be resistant to alkylating agents. In contrast, the HT-1080 and SW-872 cells were obtained from untreated patients (Ref. 13; Footnote 4).

Preparation of Trace Element and Drug Solutions. Stock solutions

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[3] The abbreviations used are: α-MEM, α-minimal essential medium; FBS, fetal bovine serum; *cis*-DDP, *cis*-diamminedichloroplatinum(II); GSH, reduced glutathione.

[4] J. Fogh, personal communication.

of $ZnCl_2$, K_2SeO_3, and $CuCl_2$ were prepared in 0.01 N HCl, sterilized by filtration, then dispensed into polyethylene tubes, and stored at −20°C. Dilutions of all 3 compounds were prepared in distilled water. The alkylating agent melphalan (also designated L-PAM, L-phenylalanine mustard, or sarcolysin), NSC 8806, was provided by Dr. D. Abraham, Investigational Drug Branch, National Cancer Institute, Bethesda, MD. Solutions of melphalan were dissolved in acidic ethanol (70% ethanol in 0.05 N HCl), sterilized by filtration, transferred to polyethylene tubes, and stored at −70°C for periods of 24 h or less; aliquots of drug were then thawed immediately prior to use.

Survival Experiments. Stock monolayer cultures were trypsinized, and a series of T75 flasks was set up, with each T75 flask containing ~8 × 10^5 cells in 25 ml of α-MEM:10% FBS. The flasks were incubated for 36 h at 37°C in a CO_2 incubator, after which the overlay medium was removed from a portion of the flasks and replaced with fresh medium (uninduced control cultures). The overlay medium was removed from the remaining flasks, and 25 ml of α-MEM:10% FBS, supplemented with either 100 μM $ZnCl_2$ or with 100 μM $ZnCl_2$, 60 μM $CuCl_2$, and 5 μM K_2SeO_3, were added back (trace element-induced cultures). Following incubation for 12 h (the induction period), the overlay medium was withdrawn from all flasks, and dilutions of freshly thawed melphalan in complete α-MEM were added. Three h later, the monolayer cultures were trypsinized, and the cells were counted, diluted, and plated in drug-free medium (200 to 6 × 10^4 cells/dish) directly into 60-mm plastic tissue culture dishes. After 12 to 14 days at 37°C in a CO_2 incubator, the plates were washed, fixed, and stained with crystal violet prior to determining the fraction of survivors (*i.e.*, colonies of 50 or more cells).

RESULTS

When zinc-induced and uninduced normal cells were exposed to different doses of melphalan and the surviving cell fractions were determined (Chart 1*A*), it was apparent that the induced culture was more resistant (*i.e.*, greater survival at any given dose of melphalan). For ease of comparing results from a number of experiments, the concentration of melphalan required to reduce survival of the uninduced culture by 3 orders of magnitude (down to the 0.1% level) was determined, and then the survival of the induced culture at that drug concentration was calculated. In Chart 1*A*, the induced cells were nearly 8 times more resistant than were their uninduced counterparts. In contrast to the results obtained with normal cells, zinc pretreatment had no effect on survival of human tumor TE-32 cells following exposure to melphalan (Chart 1*B*), indicating that the protective response was not induced in this tumor line.

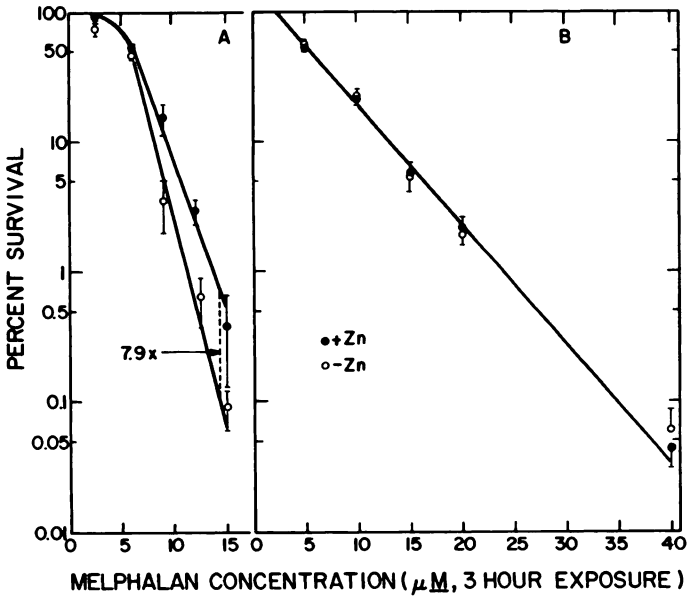

Chart 1. Effect of zinc pretreatment on survival of HSF-22 normal cells (*A*) or TE-32 tumor cells (*B*) in cultures subsequently exposed to melphalan. Induced cultures were exposed to medium containing 100 μM $ZnCl_2$ for 12 h prior to addition of melphalan-containing medium to both induced and uninduced cultures. Following a 3-h incubation period, cells were plated in drug-free medium. *Bars*, SD.

The effectiveness of zinc as an inducer of protection against melphalan toxicity is summarized in Table 1 for the entire series of normal and tumor cells. All 4 normal cells were made more resistant to melphalan (approximately 7- to 9-fold increase in survival) by pretreatment for 12 h with 100 μM $ZnCl_2$. Note also that the uninduced newborn fibroblasts appear to be more resistant than the uninduced adult fibroblasts, since higher concentrations of melphalan were required to reduce survival to the 0.1% level in newborn cells than in adult-derived cells, perhaps suggesting an age-dependent loss in resistance.

Zinc was much less effective in stimulating an increase in resistance to melphalan toxicity in the tumor cells. The largest increase in induced resistance among the tumor cell series was observed in the HT-1080 fibrosarcoma, in which survival was increased an average of only 1.7-fold by zinc pretreatment. Four of 7 tumor cell types exhibited either no increase in resistance or, in the case of SW-872 cells, a possible slight potentiation of toxicity in one experiment as a result of pretreatment with $ZnCl_2$. This failure to induce an appreciable degree of protection in tumor cells was observed in both highly resistant cells (*e.g.*, A549 and TE-32) and in melphalan-sensitive cells (*e.g.*, HT-1080 and SW-872).

The results further demonstrate that the tumor cells maintained the drug resistance properties of the initial isolates during prolonged cultivation in tissue culture. The cells HT-1080 and SW-872, known to be derived from untreated patients (and, therefore, most likely relatively sensitive to chemotherapeutic agents), exhibited a low degree of resistance to melphalan in the uninduced state that was roughly comparable to the resistance of uninduced, adult normal cells. In contrast, TE-32 cells, derived from a patient that had been treated aggressively with another alkylating agent prior to establishment of the line (11), were approximately 2.4 times more resistant to melphalan in the uninduced state than were the uninduced HT-1080 cells.

Previous studies with cultured Chinese hamster cells (18) indicated that induction with a combination of the trace elements zinc, copper, and selenium resulted in a degree of protection (~70-fold increase in survival) that greatly exceeded that achievable with any single inducer (~7-fold increase in survival). To determine whether the combination of trace elements administered to human cells is more effective than zinc alone, 2 normal fibroblasts and a pair of tumor lines (A101D and A549) were pretreated with 100 μM $ZnCl_2$, 60 μM $CuCl_2$, and 5 μM K_2SeO_3 for 12 h prior to exposure to melphalan, and aliquots were removed after 3 h for determination of survivors (Table 2). The combination of trace elements was without appreciable effect on survival of the tumor cells; however, zinc, copper, and selenium increased survival of the normal cells by approximately 16-fold or about twice that achievable with zinc alone (Table 1).

DISCUSSION

In the studies described in this paper, equivalent levels of inducers were provided to both normal and tumor cells, with the

Table 1

Effect of zinc pretreatment on survival of human normal and tumor cells exposed to the alkylating agent melphalan

Cell designation	Source of cell	Mean level of melphalan (μM) required to reduce survival to 0.1% in the non-zinc-induced culture[a]	% of survival of the zinc-induced culture at the level of melphalan yielding 0.1% survival in the uninduced culture[b]	Mean increase in survival of zinc-treated cells
Normal cells				
UNMA	Skin from chest of middle-aged male	5.0	0.76–0.90	8.3-fold
UNMC	Skin from chest of middle-aged male	8.7	0.66–0.75	7.1-fold
HSF-7	Neonate foreskin	12.6	0.58–0.75	6.7-fold
HSF-22	Neonate foreskin	12.6	0.79–0.98	8.9-fold
Tumor cells				
A101D	Melanoma	18.1	0.10	No increase
A204	Rhabdomyosarcoma	16.9	0.10–0.18	1.4-fold
A549	Lung carcinoma	53.3	0.10–0.14	1.2-fold
A1663	Bladder transitional cell carcinoma	29.5	0.10	No increase
HT1080	Fibrosarcoma	17.1	0.18–0.26	1.7-fold
TE-32	Rhabdomyosarcoma	40.8	0.10	No increase
SW-872	Liposarcoma	13.9	0.10–0.053	1.2-fold decrease

[a] Cells exposed to melphalan for 3 h prior to plating in drug-free medium for 12 to 14 days for colony formation.
[b] Cells in the zinc-treated cultures were incubated in medium containing 100 μM $ZnCl_2$ for 12 h prior to exposure to melphalan.

Table 2

Effect of combined zinc, copper, and selenium pretreatment on survival of human normal and tumor cells exposed to the alkylating agent melphalan

Cell designation	Mean level of melphalan required to reduce survival to 0.1% in the uninduced culture (μM)[a]	% of survival of the zinc:copper:selenium-induced culture at the level of melphalan yielding 0.1% survival in the uninduced culture[b]	Mean increase in survival of the trace element-induced cells
Normal cells			
HSF-7	13.7	1.30–1.95	16.3-fold
HSF-22	13.4	1.45–1.60	15.8-fold
Tumor cells			
A101D	17.5	0.1	No increase
A549	51.5	0.1–0.028	1.3-fold decrease

[a] Cells exposed to melphalan for 3 h prior to plating in drug-free medium for 12 to 14 days for colony formation.
[b] Cells in the zinc:copper:selenium-treated cultures were incubated in medium containing 100 μM $ZnCl_2$, 60 μM $CuCl_2$, and 5 μM K_2SeO_3 for 12 h prior to exposure to melphalan.

result that the normal cells yielded a greater degree of resistance to melphalan than the tumor cell populations (Tables 1 and 2). If differential responses of comparable magnitude can be induced in all types of normal tissue (both fibroblast and nonfibroblast cells) in human cancer patients, then the approach outlined may provide a novel means for selectively sparing normal tissue during alkylating agent chemotherapy.

To apply these findings to the clinic, one major problem to be overcome is possible toxicity of the induction protocol. Because we wanted to maximize the protective response in the present study, we deliberately utilized the highest tolerable doses of trace element inducers. It should be emphasized, however, that does of zinc only slightly in excess of normal serum zinc levels (20 μM) can increase survival of the Chinese hamster ovary cell line by 4.5-fold (16), leading us to expect that even small increases in serum zinc levels in humans might be sufficient to activate the protective response in normal tissue. Another major problem to be overcome if this technique is to be utilized in the clinic is the difficulty in elevating plasma zinc levels due to homeostatic regulation of this essential trace element in humans. Although humans can tolerate doses of dietary zinc that are roughly 20-fold greater than minimum daily requirements (12, 15), the resultant levels of plasma zinc are not markedly elevated. Obviously, novel protocols for zinc administration and delivery are required that will yield persistent, biologically active complexes of zinc.

Assuming that problems associated with zinc delivery can be overcome, unequal partitioning of zinc between normal and tumor tissue might further favor the preferential induction of a protective response in normal tissue. Studies with solid tumor model systems (4, 19–21, 23) suggest that serum-borne substances, such as trace elements, exhibit limited ability to penetrate into solid tumors to the internal layers of arrested but viable cells (the major source of cells for repopulation of the tumor following therapeutic intervention) that are located between the necrotic center and the actively dividing cells comprising the periphery of the tumor. In contrast, serum nutrients are readily taken up by the well-vascularized normal tissue. The net result is a preferential enrichment of administered inducers accumulating in the normal tissue.

The combination of all 3 trace elements in our human cell studies (Table 2) induced only a modest increase in resistance to melphalan over that obtained with zinc alone (Table 1). Obviously, from a clinical standpoint, any enhanced resistance of

normal tissue to alkylating agent toxicity resulting from multi-agent induction protocols must be weighed against possible side effects associated with the administration of potentially toxic compounds containing copper and selenium. The range in concentration between an essential level and one that is overtly toxic is much narrower for copper and selenium compounds than for those containing zinc, which might suggest that major emphasis be placed on zinc-only induction protocols.

Whatever the induction protocol ultimately selected, we wish to emphasize that we are not advocating a return to single (alkylating) agent therapeutic regimens. Instead, we are suggesting that the toxicity for normal tissue associated with administration of drugs, such as melphalan, might be reduced in any therapeutic regimen using alkylating agents, provided, of course, that the induction protocol does not significantly alter the efficacy of the nonalkylating agent components.

All of the above considerations suggest the feasibility of triggering a protective response specifically in normal tissue, but it is only natural to ask whether any data have been obtained with intact animals or humans that directly support this notion. We believe that one such report appeared very recently (2), although the authors in that study did not consider the type of induced protective response that we favor in their discussion of possible mechanisms. In the study of Berry *et al.* (2), fibrosarcoma-bearing mice were administered the alkylating agent *cis*-DDP with or without pretreatment with selenium (in the form of selenous acid). Injection i.p. of 2 to 4 μg of selenous acid per g prior to injection i.p. of 16 μg of *cis*-DDP per g reduced the mortality associated with administration of that level of the drug and generally enhanced the antitumor effects of *cis*-DDP. The authors interpreted their results to suggest a direct "interaction between selenium and the molecule of *cis*-DDP which prevents reabsorption by renal cells but does not impede antitumor activity."

While we have no quarrel with that interpretation, the striking similarities between their findings (2) and our own suggest that the type of trace element-induced protective response described in this paper also may have been activated in their tumor-bearing animals. Specifically, we note that: (*a*) selenous acid was administered 4 h prior to injection of the alkylating agent, suggesting the requirement for an induction period; (*b*) studies with both normal and tumor-bearing animals indicated that selenous acid pretreatment of *cis*-DDP-exposed animals reduced short-term drug-specific mortality, suggesting protection of normal tissue; and (*c*) clonogenic assays utilizing tumor cells grown *in vitro* indicated that selenous acid treatment had no measurable effect on *cis*-DDP toxicity (*i.e.*, selenium failed to induce a protective response in tumor cells). Further studies of this nature, using additional tumor species and animal hosts plus different trace element inducers, will be required to establish the validity of our proposed interpretation of the data of Berry *et al.* (2).

If, indeed, the protective response described in this paper can be activated in animals (and, by extrapolation, in humans as well), one may ask about the underlying biochemical mechanism(s) responsible for this enhanced resistance to alkylating agent toxicity. Our previous studies with alkylating agent-treated cultured cells indicate that the protective response is a complex multifactorial process (14, 16–18). Zinc treatment causes small perturbations in the rate of drug uptake (17) and efflux[5] and also appears to initiate subtle alterations in melphalan metabolism[6]; none of these processes, however, seems to contribute greatly to the induced protection.

Of greater importance, possibly, is induction of the reactive, thiol-containing species glutathione in zinc-treated responsive cells and, possibly, in selenium-treated cells as well (14). The levels of GSH and glutathione *S*-transferase activities roughly correlated with the degree of protection in zinc-induced Chinese hamster cells (14), and GSH and melphalan were shown to interact directly *in vitro*.[6] Formation of GSH:alkylating agent complexes *in vivo* could lead to detoxification through binding of the drug to the tripeptide species and prevention of interaction with sensitive cellular targets. For a discussion of the suggestive evidence linking cellular sulfhydryl content and sensitivity to alkylating agents, see the recent paper by Arrick and Nathan (1).

In view of the potential of trace elements as response modifiers in alkylating agent chemotherapy, studies are continuing on the nature of the induced mechanisms and the effectiveness of the induction protocol against different types of alkylating agents (*i.e.*, other than mustard-like compounds such as melphalan).

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