Antimicrotubule Effects of Estramustine, an Antiprostatic Tumor Drug

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ABSTRACT

Estramustine [17β-estradiol 3 n bis(2-chloroethyl)carbamate; EM] is a stable conjugate of estradiol and non-nitrogen mustard that is used for the treatment of human prostatic carcinoma. We have studied the cytotoxic effects of EM on the cytoskeletal organization of squirrelfish pigment cells (erythrophores) and human prostatic tumor cells (DU 145) in culture. Light and whole-mount electron microscopy studies reveal that, at μM levels (60 to 120 μM), EM has a dose-dependent disruptive effect on cell shape, cytoskeletal organization, and intracellular transport.

Upon removal of the drug, the cytological effects of EM are rapidly reversible in fish cells but not DU 145s. Immunofluorescent studies reveal that EM produces microtubule disassembly in fish erythrophores and DU 145 cells. A concomitant disruption of actin-microfilament arrays also occurs in DU 145 cells. These morphological data suggest that EM, in contradistinction to its constituent estradiol:nitrogen mustard species, induces cytotoxicity as an antimicrotubule drug. The observed disruption of the microtubules and cytomatrix of interphase cells is not reversible in the prostatic carcinoma cells. The disruptive action of EM on the cytoskeleton could ultimately produce a cytotoxic antimitotic effect in dividing cells.

INTRODUCTION

EM is a therapeutic agent used to treat advanced prostatic cancer (9, 10, 12). Prostatic carcinoma cells in culture (6) and more recently HeLa and Walker 256 carcinoma cells (21) have shown a sensitivity to μM levels of estramustine. The cytotoxic effects of EM in vitro are independent of the drug's constituent steroid and alkylating agent species (20) and have been linked, at least in part, with hydrophobic interactions with the structural proteins of the nucleus, the nuclear matrix (21).

It has been clearly established that no electrophilic alkylating species are produced under physiological cell culture conditions; neither does the drug express characteristics which would suggest steroid activity (for review, see Ref. 20). These properties are consistent with the relative stability of the carbamate ester bond, which links the mustard and steroid moieties (Chart 1), and suggest that pharmacological activity is mediated by the complete EM molecule.

However, a definitive explanation of the cytotoxic mechanism of EM is lacking, although much of the drug (about 75%) is known to bind other cellular components outside the nucleus (21). Consequently, it was of interest to determine possible cytomatrix effects of EM and learn how cytoplasmic related effects of EM might ultimately produce the reported antimitotic events in dividing cells (6).

In this paper, we have investigated possible cytotoxic effects of EM at the cytological level. For these studies, the fish erythrophore or red pigment cell has been used as a model system for investigation of the cytotoxic consequences of EM. There are a number of attractive reasons for utilizing erythrophores for the work described here. Erythrophores are symmetrical cells with thousands of radially ordered microtubules which control the directed motion of numerous red pigment granules (14, 19). At the light microscopic level, the pigment is observed to pulsate or to aggregate and disperse from the cell center in a cyclic fashion. Because this pigment motion is dependent upon the organizational properties of the microtubules and the elastic, contractual properties of MAP-2 rich microtubule cytomatrix complex (14, 17), the effects of EM on structural and functional properties of these protein structures can be assessed directly (14, 19). We report in this paper that EM disrupts the cytoskeletal microtubules and cytomatrix in fish erythrophores and DU 145 cells, a human prostatic carcinoma cell line. These effects are reversible in fish cells but irreversible in DU 145 cells when the drug is removed. The significance of these results to the mechanism of action of EM and its use as a chemotherapeutic agent is discussed.

MATERIALS AND METHODS

Human prostatic DU 145 tumor cells were maintained in culture as described previously (7, 18). Cells are plated at a density of 10⁶ cells/ml in 35-mm Petri dishes or on carbon-coated plastic coverslips, incubated overnight, and used the following day.

Erythrophores and epithelial cells are cultured from the scales of the squirrelfish Holocentrus ascensionis (rufus) using procedures of Stearns and Ochs (19). Freshly plucked scales are washed 6 times in sterile CM-PBS; then, the flap of skin containing epithelial cells on the scale's surface is peeled back to reveal the subdermal layer of chromatophores. After brief flushing with CM-PBS, the scales are incubated with sterile CM-PBS, containing 0.15% hyaluronidase (type II; Sigma), 0.2% colagenase (type IV; Worthington), and 1.5% bovine serum albumin (type V; Sigma) for 45 min at room temperature. Pigment cells and epithelial cells are then removed from the skin by gentle flushing with sterile CM-PBS. The cells are pelleted by centrifugation for 5 min at 6000 x g in a table-top clinical centrifuge. The cells are resuspended in Eagle's basal medium containing 15% fetal calf serum. Cultured erythrophores or DU 145 cells are grown on carbon-coated, glow-discharged coverslips. For experimental studies, cells are washed twice with fresh medium or PBS and exposed to medium or PBS at pH 7.3 and 23°C, containing EM. The drug is removed by washing cells with fresh medium or PBS. The effects of EM are recorded directly by phase-contrast microscopy of live cells or after glutaraldehyde fixation and immunolabeling for epifluorescent microscopy studies. β-Tubulin monoclonal antibodies made against pig brain tubulin were a gift of Dr. Lester I. Binder, University of Virginia, Charlottesville, VA. (2).

For electron microscopy studies, cells are fixed with 2% glutaralde-
mustard through a carbamate:ester linkage. EM functions as a drug which induces cell death without steroid activity and without alkylating activity (20).

Table 1

<table>
<thead>
<tr>
<th>EM concentration (μM)</th>
<th>Effect in min</th>
<th>Reversibility in Fish cells (DU 145)</th>
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</thead>
<tbody>
<tr>
<td>30</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>60</td>
<td>80</td>
<td>Yes Partial</td>
</tr>
<tr>
<td>120</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>240</td>
<td>15</td>
<td>No</td>
</tr>
</tbody>
</table>

Identical results were achieved when EM was applied to erythrophores still on the isolated scales of fish (i.e., not subcultured onto coverslips), except the time required for inhibition of transport was approximately doubled.

RESULTS

Cultured fish erythrophores, plated on glow-discharged and carbon-coated coverslips, attached and spread to form symmetrical, circular cells resembling erythrophores on the scales of fish. The cultured erythrophores retain an ability to aggregate metrical, circular cells resembling erythrophores on the scales of fish. The cultured erythrophores retain an ability to aggregate...
similar actin-labeling studies did not reveal any changes in microfilament arrangement in these cells.

Whole-mount electron microscopy studies show that, besides microtubules and microfilaments, DU 145 cells and erythrophores also contain a fine cytoplasmic network of filaments termed the cytomatrix network (Fig. 6) by Porter and Stearns (15). Fig. 6a shows that untreated DU 145 cells or thin, flattened cells with a fine 3-dimensional cytomatrix pervading the cytoplasm (see Fig. 6a, inset) in a pattern which resembles that observed in a number of diverse cell types (15). With EM treatment (120 μM, 20 min), a coarsening of the cytomatrix or 3-dimensional microtubule lattice component (15) occurs in both DU 145 cells (Fig. 7a) and erythrophores (not shown). Increases in the volume of the spaces between cytomatrix filaments are observed, and the cytomatrix eventually "contracts" around the cell nucleus resulting in a rounding of the cells at 20 min at 120 μM EM (Fig. 7b).

Control studies using estradiol (120 μM) on either erythrophores or DU 145 cells did not cause any of the changes in cell shape or ultrastructural organization observed with EM. In addition, nor-nitrogen mustard, although producing toxicity in both cell types at 25 μM, did not produce specific antimicrotubule effects (data not shown).

Calculated Studies. To test the possibility that calcium influences EM activity, we exposed cells to calcium:ethylenerhynoglycerol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid:PBS with increased Ca²⁺ ion levels (10⁻⁹ M Ca²⁺, 10⁻⁸ M Ca²⁺, 10⁻⁷ M Ca²⁺). EM at dosages of 120 μM had the same effect (as reported above) on erythrophans, epithelial cells, and DU 145 cells at all Ca²⁺ ion concentrations tested.

Interestingly, cold treatment did augment the effects of EM. Pretreatment of cells at 4°C for 60 min followed by exposure to 60 μM EM at 4°C for 10 min (or direct exposure of cells to 60 μM EM at 4°C for 10 min) affects cells in a manner equivalent to 120 μM EM treatment for 20 min at 23°C. The effective threshold concentration of EM is reduced to 30 μM at 4°C for 20-min treatment.

DISCUSSION

The results demonstrate that the antiprostatic tumor drug EM causes a rapid disruption of cytoskeletal organization in both fish epithelial and DU 145 cells. It is known that there is virtually no cleavage of the carbamate ester bond by cultured DU 145 cells (6). Hence, this disruption is manifested by the parent compound binding to constituent proteins of the cytoskeleton. The stability of the microtubules is immediately affected by EM, and a rapid disassembly of the tubules, apparently from their distal ends inwards, takes place. The effects of EM closely resemble the antimicrotubule drugs nocodazole, vinblastine, and colchicine (9). Nocodazole and colchicine, however, bind tubulin on a 1:1 molar basis (9) and are effective on erythrophores microtubules at low concentrations of 0.1 μM to 1.0 μM (1), whereas EM works at concentrations of approximately 60 μM at 23°C or 30 μM at 4°C. Colchicine is an approximate 90% inhibitory concentration value (6) in DU 145 cells as judged by clonogenic assay. If cell proliferation is used as a measure, the 90% inhibitory concentration value is increased to approximately 80 μM. Both values are for a 24-h incubation. Hence, the observed cytomatrix effects are obtained with a pharmacologically meaningful concentration.

Presumably, these differences in drug concentrations required to cause tubule disassembly reside with differences between the mechanism of action of EM and other antimicrotubule compounds. We propose that, in contrast to nocodazole, which binds tubulin directly (8), EM works by interaction in a "detergent-like" manner to disrupt microtubule structures, possibly by binding tubulin molecules or MAPs on the tubule surface. In other words, EM produces dissociations of the microtubule proteins or microtubule-bound cytomatrix components, indirectly resulting in microtubule disassembly. Because microtubules are less stable at low temperatures (9), lower EM concentrations are required to produce the same pharmacological effects. Our recent studies (18) using a novel fluorescent probe, dansylated EM, have shown that the drug enters DU 145 cells in vesicles, even at 4°C.

Other studies have shown that EM can inhibit the assembly, or cause disassembly, of microtubules in vitro (11). Further, EM does not compete for colchicine and nocodazole binding sites on tubulin molecules (4). Most importantly, a microtubule stabilizing agent taxol (16) prevents the EM-induced disassembly of microtubules, whereas exogenously supplied native stabilizing agents like MAPs fail to do so (11). Our interpretation is that EM may bind MAP in vivo, a conclusion which is indirectly supported by observations that pigment motility, a MAP-2-dependent function, is inhibited by EM. Preliminary work in our laboratory also indicates that EM causes MAP-2 aggregation into discreet patches along the surfaces of taxol-stabilized microtubules in cultured erythrophores.

Since cytomatrix organization and stress-fiber distributions are also simultaneously affected by EM and because these effects cannot be totally accounted for by tubule disassembly events, we believe EM may have some affinity for other cytoskeletal proteins as well. Alternatively, EM could perturb ionic balances, nucleotide levels, or phosphorylating enzymes to interfere indirectly with structural properties of protein polymers comprising the cytomatrix. We have not ruled out the possibility that EM can affect these factors and thereby produce the observed microtubule disassembly and accompanying cytomatrix and microfilament collapse. We have ruled out calcium influxes from extracellular sources as possible mechanism of EM-induced tubule disassembly.

The nonreversible nature of the effect of EM on DU 145 cells is unusual but not unexpected. Several authors have shown that prostatic tumor cells contain a M, 46,000 protein with a high affinity for EM (4, 5). It is possible that, because of this EM binding protein, EM accumulated by DU 145 cells is not released, as in the fish cells, so that the recovery of cell shape and cytoskeletal organization cannot ensue. This possible function of EM is reflected further by binding interactions within nuclear compartments. Since the nucleus and cytoplasmic matrix components have been shown to be contiguous (13), it is reasonable to conclude that the drug may influence both compartments, although EM appears to have a preferential affinity for cytoskeletal proteins (21).

From an experimental standpoint, EM provides a novel pharmacological tool with a rather specific antimicrotubule activity. The existence of such a compound with an ability to dissociate MAPs from tubules could prove to be of immense significance in cell biological research. The eventual elucidation of the biochemical mode of action of EM could certainly provide insights into...
the intricate mechanisms regulating MAPs microtubule-dependent interactions with other cell structures (e.g., 10-nm filaments, microfilaments, vesicles, and pigment granules) and their involvement in vital transport processes (18).

The "mitotic blocking" activity of EM could be explained, at least in part, by an EM-induced disorganization of vital, MAPs-triggered microtubule assembly processes. With regard to this, the differences reported here in sensitivity between interphase fish epithelial cells and malignant DU 145 prostatic cells during recovery from EM could be a favorable factor in determining the therapeutic efficacy of EM. Consideration of the antineoplastic properties of EM will reflect its reported antimitotic properties in dividing cells (7).

REFERENCES

Fig. 1. Phase-contrast pictures of a caffeine-treated (2.5 mM for 2 min) dispersed erythrophore exposed to EM (120 μM at 23°C) for 0 min (a), 5 min (b), and 20 min (c). The untreated cell exhibits evenly dispersed pigment granules (the dark material, arrows), and in response to EM, the cell begins to arborize slightly by 5 min, and "pigment-patching" (arrows) occurs as "saltatory" motion stops in the peripheral margins. By 20 min, the cells have arborized further, and pigment motion has completely stopped. Epithelial cells (EC) exhibit an extensive blebbing by 5 min, and they change shape (compare a with Fig. 2, b and c) and round-up as the cytoplasm appears to accumulate around the nucleus (N) and as the peripheral margins become transparent. d shows that EM has similar effects on erythrophores (E) and on scales. Some irridiphores (I) are visible, and they are not affected. e and f, shows that the erythrophore seen in a to c, has recovered from EM after 60 min in fresh medium. The cell recovered an ability to aggregate (e) and disperse (f) its pigment and to move its pigment in a saltatory manner. Both erythrophores and epithelial cells recover their normal shape. × 430.

Fig. 2. Glutaraldehyde-fixed erythrophores stained with β-tubulin monoclonal antibody after EM (120 μM) treatment for 0 min (a), 20 min (b), and after recovery from EM treatment, 60 min (c). Microtubule (M) staining gives the fibrillar pattern in a and c. EM treatment drastically reduces the fibrillar staining pattern (b) with only occasional microtubules remaining. The dense staining of the centriolar complex (C) and lighter nuclear staining (N) indicate the presence of tubulin in these structures. × 430.
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Fig. 3. Phase-contrast images of untreated (a) and EM-treated (b) DU 145 cells. The EM (120 μM for 20 min) produces cell rounding, vesicle formation (arrows), and an accumulation of cytoplasmic material around the nucleus. See also Fig. 5b. x 430.

Fig. 4. Glutaraldehyde-fixed DU 145 cells stained with β-tubulin antibody and fluorescein isothiocyanate:lgG. The bright microtubule pattern observed in a is not preserved following EM treatment at 120 μM for 20 min (b). Incubation of the treated cells in fresh medium for 2 h produces some random reassembly of microtubules (b), but the cells usually do not reform a normal microtubule array, and they remain rounded. x 1600.

Fig. 5. Glutaraldehyde-fixed, rhodamine-Phallacidin-stained DU 145 cells following EM treatment at 120 μM for 20 min (a). The fluorescent picture (a) shows the remaining microfilaments collected at a focus of origin (arrows) over the nucleus (N), and the phase image shows that cell rounding has occurred (b). Normally, cells exhibit numerous parallel arrays of microfilaments, but this pattern was disrupted with EM. x 430.

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Fig. 6. Whole-mount, 100-kV electron microscopic picture of DU 145 cells. Nucleus (N), mitochondria (M), filamentous cytomatrix (arrows) are shown. b shows a high magnification view of the area outlined in a. The cytomatrix consists of a cross-linking 3-dimensional network of filaments (3- to 9-nm diameter) termed the cytomatrix or microtrabecular lattice by Porter et al. (14). No microtubules or microfilament bundles are visible here. a, × 3,200; b, × 38,000.

Fig. 7. Whole-mount electron microscopy images of DU 145 cells exposed to 120 μM EM for 5 min (a) and 20 min (b). The cytomatrix coarsens in appearance by 5 min as spaces or holes in the cytomatrix increase in size (a). By 20 min, the cytomatrix contracts around the nucleus as the cell rounds up (b). Compare Fig. 6, a and b, with a. The cytomatrix in b was too thick for high-resolution images of its substructure. a, × 3,200; inset, × 25,000; b, × 3,900.
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