Antiinvasive Activity of Estramustine on Malignant MO4 Mouse Cells and on DU-145 Human Prostate Carcinoma Cells in Vitro


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ABSTRACT

Estramustine (EM) is a conjugate of estradiol and nor-nitrogen mustard (nor-HN2), which is effective in the treatment of prostate cancer. We have compared the effect of EM with that of the known microtubule inhibitor vinblastine (VLB) on the following functions of malignant MO4 mouse cells and of DU-145 human prostate cancer cells in vitro: directional migration, invasion; and the organization and the assembly/disassembly equilibrium of microtubules. The circular area covered by cells migrating from an aggregate explanted on a solid substrate was taken as an index of directional migration. Invasion was studied through confrontation of MO4 or DU-145 cells with fragments of embryonic chick heart in organ culture. Microtubules were investigated immunocytochemically and through immunodetection on protein blots. VLB and EM inhibited directional migration and invasion of MO4 and DU-145 cells in a dose-dependent manner; equimolar combinations of estradiol plus nor-nitrogen mustard did not mimic these effects. At antiinvasive concentrations VLB led to partial disassembly of microtubule complexes, whereas EM resulted in an abnormal pattern of microtubule complexes without alteration of the overall assembly/disassembly equilibrium. Combined treatment with VLB and EM resulted in an enhanced VLB effect, namely complete disassembly. In all tests DU-145 cells were more sensitive to both VLB and EM than were MO4 cells, and the effects were less reversible. The present experiments showed that EM shares an antiinvasive activity with other microtubule inhibitors.

INTRODUCTION

EM is a steroid-alkylating agent, consisting of estradiol conjugated to nor-HN2 through a carbamate ester linkage. EM is a major metabolite of Estracyt (AB Leo, Helsingborg, Sweden), a drug effective in the treatment of advanced prostate cancer (1, 2). Originally, the anticancer activity of EM was ascribed to its constituents estradiol and nor-HN2 (for review, see Ref. 3). Our interest in EM comes from three recent aspects of its mechanisms of action: (a) EM per se was found to be more potent than estradiol plus nor-HN2 in inhibition of proliferation of cultured cells (4, 5); (b) there exists a naturally occurring estramustine-binding protein (6); (c) the following observations indicated that EM acted as a microtubule inhibitor: (a) increase of the mitotic index through metaphase arrest in cultured cells (5); (b) microtubule disassembly in squint-fish erythrocytes and in DU-145 cells (7); (c) interference with fast axonal transport in isolated nerve (8); (d) inhibition of microtubule assembly and induction of disassembly of isolated brain microtubules (8); (e) binding to MAPs (9). The latter finding suggested that the mechanism of action of EM on MTCs was different from that of the majority of microtubule inhibitors that act through binding to tubulin.

We have found that microtubule inhibitors stop tumor invasion and that this antiinvasive activity is not dependent upon the way these agents interact with the assembly/disassembly equilibrium of microtubules. For example, an antiinvasive activity was found for colchicine-like agents that lead to microtubule disassembly as well as for taxol that leads to overassembly of microtubules (reviewed in Ref. 10). It was our aim to find out whether EM would share antiinvasive activity with other microtubule inhibitors tested so far (listed in Ref. 11). Therefore, we have examined (a) the effect of EM on invasion of malignant mouse cells (MO4) in organ culture, a process that is sensitive to microtubule inhibitors (12), (b) the effect of EM and of the well-documented microtubule inhibitor VLB on the invasion of human prostate carcinoma cells (DU-145) in organ culture not tested for sensitivity to antiinvasive agents so far, and (c) the effect of EM and VLB and of combinations of both on the organization of MTCs and on the microtubule assembly/disassembly equilibrium in MO4 and DU-145 cells by immunocytochemistry and by immunodetection on protein blots using an antisera against tubulin.

MATERIALS AND METHODS

Cell Lines. The MO4 cell line was derived from an immortalized C3H/He fetal mouse cell line after transformation by Kirsten murine sarcoma virus (13). MO4 cells are invasive in vitro (14) and metastatic in vivo (15).

The DU-145 cell line was isolated from a brain metastasis of a human prostate carcinoma (16). We obtained this cell line from Dr. Don Mickey (Duke University Medical Center, Durham, NC) at passage 65 in 1979. DU-145 cells were used in the present experiments between passages 99 and 110. The cell lines were maintained on 25-cm² tissue culture substrate with minimum essential medium Eagle's (modified) with Earle's salts and nonessential amino acids (Flow Laboratories, Irvine, Scotland) for MO4, and RPMI 1640 (Gibco Limited, Paisley, Scotland) for DU-145, both supplemented with 10% (v/v) fetal bovine serum, 0.05% (w/v) l-glutamine, 250 IU/ml penicillin, and 100 µg/ml streptomycin (hereafter called culture medium).

Drugs. EM [1β-estradiol, 3-[N,N-bis(2-chloroethyl)carbamate]]; M, 440.41; synthesized at AB Leo and 17β-estradiol; M, 272.37; purchased from Diosynth B.V., Oss, The Netherlands were dissolved in absolute ethanol at 1 and 5 mg/ml, respectively. Nor-HN2 (synthesized at AB Leo; M, 141.8) was dissolved in distilled water at 0.5 mg/ml. VLB (Vinca leuckoblastine; M, 811.00; obtained from Eli Lilly Benelux, Brussels, Belgium) was dissolved in Ringer's balanced salt solution at 1 mg/ml. Stock solutions were stored at −25°C and diluted in culture medium immediately before use. Control cultures contained equivalent amounts of solvent.

Assay for Directional Migration. Multicellular spheroids of MO4 or DU-145 cells were prepared by incubation of a single cell suspension on a Gyratory shaker (New Brunswick Scientific Company Inc., New Brunswick, NJ) as described earlier (14). Spheroids with a diameter of
0.2 mm were selected under a MacroScope (Wild, Heerbrugg, Switzerland) and explanted on glass (for MO<sub>4</sub> spheroids) or on tissue culture plastic substrate (for DU-145 spheroids) as individual cultures. DU-145 cells attached poorly to glass. After attachment of the spheroid to the substrate, cells started to migrate radially from the spheroid and the diameter of the circular area covered by the cells was taken as an index of directional migration (17). The direction of migration is mediated through contact inhibition of ruffling and loss of contact inhibition results in random migration with reduced increase in the area covered by the cells (18). Drugs were added after the onset of directional migration. We know from previous experiments that inhibition of cell proliferation does not affect the index of directional migration during the first 4 days of culture (17, 19). So far, a good correlation was found between drug-induced inhibition of directional migration and inhibition of invasion (10).

Assay for Invasion. Spheroids of MO<sub>4</sub> cells and of DU-145 cells (diameter, 0.2 mm) were confronted with precultured fragments of 9-day old embryonic chick heart (PHF; diameter, 0.4 mm) as described earlier (14). Briefly, a spheroid was put into contact with a PHF on top of a semisolid agar medium. After attachment of both partners to each other the confronting pair was transferred into liquid medium and further incubated on a Gyrotory shaker for 4 days. Then, the confronting pair was fixed in Bouin-Holland's solution and processed for embedding in paraffin. The whole pair was sectioned into 8-μm thick serial sections. Consecutive sections were stained with hematoxylin-eosin or with an antiserum against chick heart (20). Invasion was scored by two independent observers on the basis of occupation and replacement of the heart tissue following a subjective grading (21).

The relevance of this assay in vitro for the present investigation is supported by a good correlation between invasion in the assay and invasion in vivo (22) and by the finding that inhibitors of invasion in vitro were antiinvasive also in vivo (23).

Immunocytochemical Assay of Tubulin. Cell suspensions freshly trypsinized from stock cultures were seeded at a concentration of 1 × 10<sup>4</sup> cells/ml (MO<sub>4</sub>) on round glass coverslips (diameter, 15 mm) or at 3.5 × 10<sup>4</sup> cells/ml (DU-145) on glass coverslips coated with collagen type IV (1 mg/ml; Sigma, St. Louis, MO). Cells on coverslips were incubated at 37°C in a 24-well multidish (Nunc, Roskilde, Denmark) containing 1 ml culture medium/well for 3 days prior to drug administration. Drugs were added at concentrations from noneffective to toxic and in different combinations for 3 h. Reversibility after drug treatment was evaluated as follows: cultures were washed three times with 1 ml drug-free medium at room temperature and reincubated at 37°C for 3 or 20 h. Cultures incubated for 20 h were washed once more after 3 h. For immunocytochemistry, cells were fixed immediately (without washing) in 1% glutaraldehyde (in 0.1 M cacodylate buffer) for 10 min at room temperature, treated with 0.1% (w/v) NaBH<sub>4</sub> in ethanol:distilled water, 1:1, and stained with an antiserum against tubulin (kindly provided by Marc De Brabander and Jan De Mey, Janssen Pharmaceutica, Beerse, Belgium) according to the peroxidase-antiperoxidase procedure described by De Mey et al. (24). Immunocytochemical preparations were scored by two independent observers on coded slides.

Immunodetection of Tubulin on Protein Blots. We seeded 5 × 10<sup>5</sup> MO<sub>4</sub> cells on 25-cm<sup>2</sup> tissue culture flasks. After 4 days of incubation at 37°C cultures were treated with VLB, or with EM, or with a combination of both for 3 h. Then, cultures were washed twice in Dulbecco's phosphate buffered saline and treated in a microtubule stabilizing buffer for the preparation of extractable and cytoskeletal proteins in accordance with the method of Solomon et al. (25). As demonstrated by these authors, the extractable fraction contains the unassembled tubulin, whereas the cytoskeletal fraction contains tubulin from assembled microtubules. Monodimensional SDS-polyacrylamide gel electrophoresis (26) was carried out in a 10.5% (w/v) separating gel. Sample preparation was in 1.3% (w/v) SDS, 3.5% (v/v) 2-mercaptoethanol, and 50 mm 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES) buffer. After heating at 95°C for 5 min, insoluble material was removed by centrifugation. Prior to electrophoresis the samples were diluted 1:2 to 1:6 in 0.062 M Tris-HCl buffer (pH 6.8), 2% (w/v) SDS, and 1% (v/v) 2-mercaptoethanol, in accordance with the results obtained with immunocytochemistry. The applied volume of the sample solution was 20 μl/lane. After SDS-polyacrylamide gel electrophoresis the proteins were blotted onto nitrocellulose sheets (Hybond-C; Amersham, International plc, Buckinghamshire, United Kingdom). The electrophoretic transfer was performed overnight at 200 mA and 50 V (27). Blocking of the remaining free sites was done in phosphate buffered saline containing 0.5% (v/v) Tween 20. The primary antibody was antitubulin, as used for immunocytochemistry, diluted 1:250, supplemented with 1% (v/v) normal goat serum and 5% (w/v) fat-free dry milk. Immunodetection was done in accordance with Blake et al. (28) using anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1:750. According to the latter authors this method provides quantitative data over a wide range of antigen concentrations. Quantitation of blots was done by image analysis (Vicom Inc., San Jose, CA).

RESULTS

Assay for Directional Migration. Directional migration of MO<sub>4</sub> cells from an explanted aggregate was inhibited after addition of 5.9 μg EM/ml (Fig. 1). The inhibition was clearly seen 1 day after addition of the drug and was found to be reversible within the first day after washing of the cultures with drug-free medium. A similar curve was obtained with VLB and with other Vinca alkaloids (29). A slight inhibitory effect was also noted for the combined treatment with estradiol plus nor-HN2 at concentrations equimolar to 5.9 μg EM/ml.

To determine concentrations of VLB useful for invasion experiments we used the same assay and found that the directional migration of DU-145 cells was sensitive to VLB at concentrations between 0.001 and 0.01 μg/ml (Fig. 2). The effect of EM at 5.0 μg/ml was similar to that of VLB at 0.01 μg/ml or higher, whereas cultures treated with combinations of estradiol (up to 42 μg/ml) plus nor-HN2 (up to 7.4 μg/ml) hardly differed from control cultures (data not shown). A dose-response curve of the sensitivity of DU-145 cell directional migration to EM is shown in Fig. 3. The data indicate that EM inhibits directional migration of DU-145 cells at concentrations between 1 (no effect) and 5 μg/ml (maximal effect). Recovery from the inhibitory effect of EM on the directional migration of DU-145 cells appeared only 4 to 6 days after removal of the drug (Fig. 4). This was explained by phase contrast microscopy of living cultures that showed necrosis of a number of DU-145 cells.
ANTIINVASIVE EFFECT OF EM

Table 1 Effects of estradiol plus nor-HN2 and EM on invasion of MO4 cells in organ culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4 days</th>
<th>8 days</th>
<th>4 + 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>IV (5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CH3CH2OH (0.2%)</td>
<td>IV (5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Estradiol (3.7 µg/ml) + nor-HN2 (2.2 µg/ml)</td>
<td>IV (5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EM (5.9 µg/ml)</td>
<td>II (5)</td>
<td>II (5)</td>
<td>III (5)</td>
</tr>
</tbody>
</table>

* Grades I or II, confronting cells were at the periphery of the heart tissue (no invasion); Grades III and IV, confronting cells had occupied and/or replaced less than or more than one-half of the PHF, respectively (21).
* Four days with drug, followed by washing and 4 days without drug.
* Numbers in parentheses, number of cultures.
* ND, not done.

DU-145 cells extensively invaded the heart tissue within 4 days after confrontation with PHF (Fig. 5), and were, therefore, appropriate for antiinvasive drug testing. At 0.005 μg/ml VLB inhibited the invasion of DU-145 cells, and this inhibition was reversible. Higher concentrations of VLB were so toxic for DU-145 cells as to kill the cells, allowing no recovery studies. Inhibition of invasion was demonstrated with EM at concentrations between 1.0 and 4.0 µg/ml (Table 2). In some of these cultures invasion apparently occurred despite mitotic arrest since no postmetaphase figures were found on serial sections and blocked DU-145 metaphase figures were present inside the heart tissue (Fig. 5, E and F). At concentrations between 1.0 and 4.0 µg/ml recovery from inhibition was obvious 4 days after washing. At a concentration of 5.9 µg EM/ml invasion was absent in all cultures and relatively few DU-145 cells remained at the periphery of the PHF (Fig. 5, G and H); recovery of DU-145 cells from EM effects could not be demonstrated. Combined treatment with estradiol plus nor-HN2 had no effect on invasion by DU-145 cells (Table 2 and Fig. 5, C and D).

Immunocytochemical Assay for Tubulin. Untreated MO4 cells showed a normal CMTC with microtubules radiating in a regular array from the perinuclear cytocenter towards the periphery of the cell (Fig. 6A); mitotic cells had a normal SMTC as described previously (30). Treatment with VLB at 0.1 µg/ml for 3 h reduced the number of microtubules (Fig. 6B) and led to complete disappearance of microtubules at 0.3 µg/ml. Disassembly of microtubules by VLB was also demonstrated by immunodetection of extractable (unassembled) and cytoskeletal (assembled) tubulin on protein blots (Fig. 7). CMTCs (Fig. 6C) and SMTC were restored to normal after washing and further incubation without drug. After treatment with EM at 5.9 µg/ml...
Fig. 5. Photomicrograph of paraffin sections from confrontations between DU-145 cell (D) spheroids and embryonic chick heart (H) fragments in organ culture in presence of 0.2% (v/v) ethanol (A and B), 3.7 µg estradiol/ml plus 2.2 µg nor-nitrogen mustard/ml (C and D), 2 (E and F) and 5.9 µg estramustine/ml (G and H). All cultures were fixed after 4 days and sections were stained with hematoxylin-eosin (A, C, E, and G) or with an antiserum against chick heart (B, D, F, H). Arrowheads, blocked metaphase figures inside the heart tissue; scale bars, 50 µm.
Table 2 Effects of estradiol plus nor-HN2, EM, and VLB on invasion of DU-145 cells in organ culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Grading* after 4 days</th>
<th>Grading* after 4 + 4 days</th>
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</thead>
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<tr>
<td>None</td>
<td>IV (3), III (11)</td>
<td>IV (4)</td>
</tr>
<tr>
<td>CH3CH2OH (0.2%)</td>
<td>III (5)</td>
<td>Not done</td>
</tr>
<tr>
<td>Estradiol (3.7 μg/ml) + nor-HN2 (2.2 μg/ml)</td>
<td>III (5)</td>
<td>Not done</td>
</tr>
<tr>
<td>VLB (0.005 μg/ml)</td>
<td>II (4)</td>
<td>III (2), IV (2)</td>
</tr>
<tr>
<td>VLB (0.01 μg/ml)</td>
<td>II (4)</td>
<td>II (3)</td>
</tr>
<tr>
<td>VLB (0.01 μg/ml)</td>
<td>II (5)</td>
<td>Not done</td>
</tr>
<tr>
<td>EM (0.5 μg/ml)</td>
<td>III (4)</td>
<td>III (1), IV (3)</td>
</tr>
<tr>
<td>EM (1.0 μg/ml)</td>
<td>II (2), III (1)</td>
<td>IV (1)</td>
</tr>
<tr>
<td>EM (2.0 μg/ml)</td>
<td>II (1), III (3)</td>
<td>IV (2)</td>
</tr>
<tr>
<td>EM (3.0 μg/ml)</td>
<td>II (1), III (3)</td>
<td>III (1), IV (2)</td>
</tr>
<tr>
<td>EM (4.0 μg/ml)</td>
<td>II (1), III (2)</td>
<td>0 (2), IV (1)</td>
</tr>
<tr>
<td>EM (5.9 μg/ml)</td>
<td>II (5)</td>
<td>II (2), 0 (3)</td>
</tr>
</tbody>
</table>

* Grade 0, confronting cells were absent; Grades I or II, confronting cells were at the periphery of the heart tissue (no invasion); Grades III and IV, confronting cells had occupied and/or replaced less than or more than one-half of the PHF, respectively (invasion) (21).

Four days with drug, followed by washing and 4 days without drug.

Numbers in parentheses, number of cultures.

and sometimes interconnecting chromosomes. The immunocytochemical data did not indicate EM-mediated disassembly of microtubules as described with VLB (compare Fig. 6, B with D); this was confirmed by immunodetection on protein blots (Fig. 7). A reduced number of microtubules was observed when the concentration of EM was increased to 11 μg/ml, but further increase up to 47 μg/ml did not result in disappearance of all microtubules. All EM-induced alterations of MTCs in MO4 cells returned to normal 3 h after washing. Combined treatments with VLB and EM at concentrations that in single treatment, respectively, led to reduced and abnormal MTCs, resulted in complete disassembly (Fig. 7) with diffuse staining of unassembled tubulin only (Fig. 6E). Reversibility of the combined treatment was somewhat slower than that of single treatments. Recovery from VLB (0.3 μg/ml) treatment, leading to complete microtubule disassembly, in presence of EM (5.9 μg/ml) produced abnormal MTCs as described for EM alone (compare Fig. 6, D with F). Results of immunocytochemical data from single and combined treatments of MO4 cells are summarized in Fig. 8.

Results obtained with DU-145 cells are illustrated in Fig. 9 and summarized in Fig. 10. DU-145 cells were sufficiently spread to reveal normal MTCs (Fig. 9A) as described in MO4 cells (see Fig. 6A). At 0.01 μg/ml VLB reduced the number of

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Fig. 6. Immunocytochemical staining with antiserum against tubulin of MO4 cells cultured on glass. A, untreated, normal CMTCs; B, treated with 0.1 μg VLB/ml, reduced number of microtubules; C, treated with 0.3 μg VLB/ml followed by washing and further incubation without drugs, normal CMTCs as in A; D, treated with 5.9 μg EM/ml, abnormal CMTCs; E, treated with 0.1 μg VLB/ml plus 5.9 μg EM/ml, absence of microtubules and diffuse cytoplasmic staining; F, treated with 0.3 μg VLB/ml followed by washing and further incubation with 5.9 μg EM/ml, abnormal CMTCs as in D. All treatments were for 3 h. Scale bars, 20 μm. Symbols, status of microtubules as used in Fig. 8.
resulted in disturbed MTCs. The reversibility of the SMTCs remained abnormal. Combined treatments for another 17 h produced cells with normal CMTCs, but part of the SMTCs remained abnormal. Combined treatments with EM produced abnormal MTCs (Fig. 9D) whereas higher concentrations (10 to 30 μg/ml) reduced the number of microtubules (Fig. 9B) and led to complete disappearance of microtubules (Fig. 9C). CMTCs but not SMTCS returned to normal 3 h after washing (see Fig. 10). Lower concentrations of EM (2.0 to 5.0 μg/ml) produced abnormal MTCs (Fig. 9D) whereas higher concentrations (10 to 30 μg/ml) reduced the number of microtubules (Fig. 9E) as described also for MO4 cells (compare Figs. 8 and 10). With lower concentrations of EM both CMTCs and SMTCs were normal 3 h after washing. With higher concentrations of EM both normal and abnormal MTCs were found 3 h after washing (Fig. 10). Further incubation in drug-free medium for another 17 h produced cells with normal CMTCs, but part of the SMTCs remained abnormal. Combined treatments caused disassembly, as described for MO4 cells. The reversibility after combination of VLB plus EM was slower and less complete than for each drug alone. As for MO4 cells, recovery from VLB (0.03 μg/ml) treatment in the presence of EM (2.0 μg/ml) resulted in disturbed MTCs.

**DISCUSSION**

We found that human prostate carcinoma cells DU-145 were invasive in vitro and that their invasiveness was sensitive to the microtubule inhibitor VLB, as was shown for other cell types (10). EM, like VLB, was antiinvasive for both MO4 and DU-145 cells.

Although the responses of both MO4 and DU-145 cells to the microtubule inhibitors VLB and EM were essentially the same, differences in sensitivity between both cell types were noted. Complete inhibition of invasion by VLB was achieved at a lower concentration for DU-145 cells (present data) than for MO4 cells (29). Recovery from inhibition of invasion was more obvious for MO4 than for DU-145 cells (compare Tables 1 and 2). It is unlikely that the differences in recovery between MO4 and DU-145 cells can be ascribed to the presence in DU-145 cells of the estramustine-binding protein (about 20 ng/mg protein) for two reasons: (a) differences hold true for both EM and VLB, whereas estramustin-binding protein is highly specific for EM (31); (b) the effect of VLB and EM on CMTC was readily reversible in both types of cells. We tentatively ascribe the abovementioned differences to the ability of the cells to survive an abortive mitotic phase (30). MO4 cells resume normal mitotic activity after metaphase block (30), whereas DU-145 cells blocked in metaphase become necrotic (5). The molecular basis of this differential behavior between various types of cells is unknown. The practical consequence, however, is that we could demonstrate that inhibition of invasion by EM or by VLB was not due to cytotoxicity quite convincingly for MO4 cells, but less so for DU-145 cells.

The antiinvasive activity of EM was due to the complete molecule and not to its constituents estradiol and/or nor-HN2. The arguments are (a) combined treatment with estradiol plus nor-HN2 at concentrations equimolar to an antiinvasive concentration of EM was ineffective (see Table 2), and (b) Hartley-Asp and Gunnarsson (32) have shown that in culture the carbamate ester bond between estradiol and nor-HN2 remains intact.

Antiinvasive concentrations of VLB and EM also interfered with directional migration and affected the organization (EM) or the assembly-disassembly equilibrium (VLB) of the CMTC. This is in agreement with the conclusion from previous experiments that inhibition of invasion is due to functional disturbance of the CMTC leading to interference with directional migration (for discussion, see Ref. 10). Inhibition of invasion was not ascribed to interference with cell proliferation through alteration of the SMTC since antiproliferative agents that left MTCs intact permitted invasion (11, 29). One of the present observations supports this conclusion: in some cultures 2 μg EM/ml plus 5.9 μg EM/ml produced normal CMTCs, but part of the SMTCs remained abnormal. Combined treatment of EM plus VLB enhanced VLB induced disassembly.

**Immunodetection of Tubulin on Protein Blots.** Immunodetection of extractable and cytoskeletal tubulin on protein blots (see Fig. 7) from MO4 cell cultures confirmed the main immunochemical data in a quantitative way (Table 3). EM, at a concentration that inhibited invasion and directional migration, did not alter the overall assembly-disassembly equilibrium, in contrast with VLB which caused disassembly. Combined treatment of EM plus VLB enhanced VLB induced disassembly.
Table 3 Tubulin assembly/disassembly equilibria calculated from immunostained blots of proteins from MO, cells treated with VLB and EM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extractable</td>
</tr>
<tr>
<td>None</td>
<td>61</td>
</tr>
<tr>
<td>VLB (0.1 µg/ml)</td>
<td>87</td>
</tr>
<tr>
<td>EM (5.9 µg/ml)</td>
<td>66</td>
</tr>
<tr>
<td>VLB (0.1 µg/ml) + EM (5.9 µg/ml)</td>
<td>96</td>
</tr>
</tbody>
</table>

Our immunocytochemical data on changes of the CMTC induced by microtubule inhibitors suggest that EM affects the microtubule assembly-disassembly equilibrium in a way different from that of VLB. Whereas VLB causes progressive disassembly in a dose-dependent manner, antiinvasive concentrations of EM cause an abnormal pattern of microtubules without alteration of the overall equilibrium. This abnormal pattern could hardly be ascribed to shape changes of the cells as shown previously by optical diffractometry and Fourier analysis (34). Higher concentrations of EM that are not relevant for their antiinvasive effect but could be helpful to understand its mechanism of action led to a reduction in the number of microtubules in accordance with other observations (7), but not to complete disassembly as observed with VLB. It is unlikely that the abnormal pattern of microtubules induced by EM represents incipient disassembly for two reasons: (a) immunoblots did not show a reduced amount of assembled tubulin; (b) when after VLB-induced disassembly microtubules were allowed to reassemble in the presence of EM the same abnormal pattern was observed, indicating that EM allows some kind of microtubule assembly. One explanation for both the disturbed microtubule assembly in the presence of EM and the complete disassembly with combinations of EM plus VLB is offered by the concept of dynamic instability of individual microtubules (35). The concept is based on real-time video recordings of isolated single microtubules and states that growing and shortening microtubules coexist under steady-state conditions in the absence of MAPs. MAPs would stabilize microtubules in the growing phase. It has been demonstrated quite convincingly that EM binds to MAPs presumably at tubulin binding sites (9, 36), making them inaccessible for binding to tubulin. This would then permit the shortening phase of microtubules to occur, causing length fluctuations that might explain the abnormal pattern. Since under these circumstances the microtubule length loss is balanced by elongation in the growing phase, the overall assembly-disassembly ratio remains constant, as shown by our...
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ACKNOWLEDGMENTS

ImmunobLOTS. In combination with VLB the growing phase of microtubules would also be prevented due to binding of VLB to tubulin, so that an unbalanced shortening phase would lead to complete disassembly.

We conclude that at least in vitro, EM has an antiinvasive activity on malignant mouse MO cells and on human DU-145 prostate carcinoma cells which, at least for MO cells, can be ascribed to structural and functional alterations of the CMTc.

REFERENCES


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