Antiinvasive Effect of Racemic 1-O-Octadecyl-2-O-methylglycerol-3-
phosphocholine on MO4 Mouse Fibrosarcoma Cells in Vitro

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ABSTRACT

Alkyl-lysophospholipids have been shown to possess antitumoral activity in animal and in human tumors. Among them, racemic 1-O-octadecyl-2-O-methylglycerol-3-phosphocholine (ET-18-OCH3) had an antimetastatic effect in experimental tumors. We investigated the effect of ET-18-OCH3 on invasion of MO4 mouse fibrosarcoma cells and on cellular activities possibly related to invasion in vitro. Ten μg of ET-18-OCH3 per ml permitted growth of MO4 cells to about 75% of controls and slightly reduced trypan blue exclusion. Directional migration inferred from the area covered by MO4 cells that had migrated from an aggregate on glass was not affected. Reassembly of microtubules after treatment with 1 μg of Nocodazole per ml occurred normally in presence of ET-18-OCH3. Invasion was completely inhibited when MO4 cell aggregates were confronted with pre-cultured fragments of embryonic chick cardiac muscle or with fresh embryonic chick lung fragments in culture on gyratory shaker in fluid medium with 10 μg of ET-18-OCH3 per ml. These experiments showed that ET-18-OCH3, in contrast with microtubule inhibitors, interfered with invasion of MO4 cells in vitro at concentrations that permitted growth and directional migration of MO4 cells. Fluorescence polarization studies with the lipophilic probe diphenylhexatriene indicated that the antiinvasive effect of ET-18-OCH3 was accompanied by an overall increase of membrane fluidity. We tentatively concluded that alterations of the MO4 cellular membranes are responsible for the antiinvasive effect of ET-18-OCH3.

INTRODUCTION

ALPs3 are ether analogues of the naturally occurring 2-LPC which have been synthesized (3, 38) to obtain a new class of biological response modifiers (27). Ethers with long aliphatic side chains in sn-1 of the molecule (for example, ET-18-OCH3) possess antitumoral activity in animal and possibly also in human tumors (6, 10, 26). Several mechanisms have been proposed for the antitumoral activity of ALP: stimulation of cytotoxic macrophages (6, 26); direct cytotoxic effects on the tumor cells (2, 7, 29, 34); and induction of cell differentiation (14).

Inhibition of tumor invasion has been recently proposed as one of the mechanisms of action of some antitumoral agents (19). All these agents belonged to the group of microtubule inhibitors which were shown to inhibit invasion in vitro (17, 18, 21, 23) and metastasis in vivo (4). The experiments with microtubule inhibitors led to the conclusion that an intact cytoplasmic microtubule complex was necessary for invading cells because it mediated directional migration. It is, however, unlikely that directional migration is the only activity required for invasion. In view of our search for other activities of invading cells, we were interested in ALP for 2 reasons: (a) ALPs were shown to inhibit metastasis from Lewis lung carcinomas in mice (5, 6, 26); and (b) considering the known mechanisms of action of ALP (5, 26), it was unlikely that it would affect microtubules. Therefore, we have examined the effect of ET-18-OCH3 on growth, directional migration, microtubule assembly, and invasion in vitro of MO4 mouse fibrosarcoma cells. Since ALP could possibly affect the composition and fluidity of membrane lipids, we have also estimated the effect of ET-18-OCH3 on the lipid fluidity of MO4 cell membranes by the fluorescence polarization technique (35–37).

MATERIALS AND METHODS

Drugs. ET-18-OCH3 (Medmark GmbH; Munich-Guenwald, Federal Republic of Germany) was dissolved in the culture medium at concentrations between 1 and 30 μg/ml. Lipid purity was assessed by thin-layer chromatography.4 Solutions were sterilized by micropore filtration (0.22 μm; Millex, Millipore, Molsheim, France) and eventually stored at −20 °C. Chemical structures of these compounds are described by Berdel et al. (5). The synthetic microtubule inhibitor (11) Nocodazole (Janssen Pharmaceuticals, Beerse, Belgium) was dissolved in dimethyl sulfoxide at 5 mg/ml and further diluted in cultured medium to 1 μg/ml.

MO4 Cells. MO4 cells were virally transformed C57 mouse cells which produced invasive and metastasizing sarcomas after transplantation into syngeneic mice (24). MO4 cells were maintained in minimum Eagle's medium with nonessential amino acids (Rega 1; Gibco Europe, Paisley, Scotland) supplemented with 10% fetal calf serum and 0.05% glutamine. This medium was used in the present experiments unless stated otherwise. Aggregates of MO4 cells were prepared by incubating a suspension of trypanized cells on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 70 rpm, as described previously (17). Aggregates with a diameter of 0.2 or 0.4 mm were selected under a stereomicroscope (>50) and washed 4 times to discard loose cells before use in the present assays.

Assays for Growth and Cytotoxicity. Growth was evaluated by daily measurements of the diameter of MO4 cell aggregates incubated individu-

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evaluate the cytotoxic effects of the drugs.

Concentrations of ET-18-OCH₃, MO4 cell aggregages with 0.4-mm di

Assay for Directional Migration. The mean diameter of the circular

Assay for Invasiveness in Vitro. MO4 cell aggregates (diameter, 0.2

Immunocytochemistry with Antiserum against Tubulin. MO4 cells

AntiiInvasiveness of ET-18-OCH₃

RESULTS

Growth and Cytotoxicity. The antiproliferative and cytotoxic

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Chart 2. Effect of ET-18-OCH₃ (O) and 2-LPC (x) at concentrations between 1

Fluorescence Polarizations Measurements. MO4 cells were har

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Directional Migration and Morphology. The effect of ET-18-OCH₃ on the directional migration of MO₄ cells from an aggregate explanted on glass is shown in Chart 3. These experiments indicated that 10 µg of ET-18-OCH₃ per ml did not affect directional migration.

Histological examination of cultures fixed after 7 days (Fig. 1) showed morphological effects of the drug at concentrations of 3 µg/ml or higher. Large polygonal polynucleated cells sometimes vacuolized were visible in cultures treated with 3 µg of ET-18-OCH₃ per ml, and their number increased with increasing drug concentrations. The presence of these large cells appeared to partly disturb the radial orientation of spindle-shaped cells that was typical of the corona in control cultures. In most cultures treated with 30 µg of ET-18-OCH₃ per ml, postmetaphase figures were absent. However, in 2 of 6 cultures, a focus of apparently unaffected cells with normal mitotic figures was observed (Fig. 1D) suggesting the presence of a subpopulation of MO₄ cells resistant to this concentration of ET-18-OCH₃.

Immunocytochemistry with Antiserum against Tubulin. Staining of MO₄ cells seeded on coverslips in absence of drug showed both spindle and cytoplasmic microtubule complexes. After 2-hr treatment with 1 µg of nocodazole per ml, microtubules were absent (Fig. 2A). When nocodazole was removed, cytoplasmic microtubule complexes reappeared within 2 hr regardless of whether cells were further incubated in control medium (Fig. 2B) or in medium containing 30 µg of ET-18-OCH₃ per ml (Fig. 2C).

Invasion into Chick Cardiac Muscle and Lung. In all control cultures (untreated or treated with 10 or 30 µg of 2-LPC per ml), using either cardiac muscle or lung MO₄ cells met the criteria of invasiveness as outlined in "Materials and Methods" (Figs. 3, A and B, and 4A). ET-18-OCH₃ at concentrations of 10 and 30 µg/ml inhibited invasion as the aggregate of MO₄ cells remained at its pole of attachment to the fragment of normal tissue (Figs. 3, C and D, and 4B). In some cultures, a few MO₄ cells were found between the peripheral cardiac muscle cells or inside the connective tissue of the lung. Partial inhibition of invasion was suspected from the histology of cultures treated with 6 µg of ET-18-OCH₃ per ml, in some of which MO₄ cells occupied a limited area of the normal tissue. Confronting cultures treated with 3 µg of ET-18-OCH₃ per ml were not different from controls. When MO₄ cell aggregates with a diameter of 0.4 mm were used, inhibition of invasion was obvious as with smaller aggregates (diameter, 0.2 mm).

Membrane Lipid Fluidity. Fluorescence polarization measurements with probe DPH have been executed on MO₄ cells which have grown for up to 3 days in the presence of 3, 10, and 30 µg µg of ET-18-OCH₃ per ml, respectively, as compared to control MO₄ cells cultured in the absence of this drug, or in the presence of naturally occurring 2-LPC (10 µg/ml). Chart 4 shows that there is very little difference between the P values of cells grown with 3 µg of ET-18-OCH₃ or with 10 µg of 2-LPC per ml, as compared to control cells. However, a significant decrease in polarization was observed, during a 3-day period of growth, in the cells with 10 µg of ET-18-OCH₃ and even more so with 30 µg of ET-18-OCH₃ per ml. This decrease was already obvious after 1 day.

It should be noted that 10 µg of ET-18-OCH₃ per ml only minimally affects cell viability and cell growth (20% reduced at most), but that 30 µg/ml reduces these parameters significantly (see also Chart 2). It is well known that the possible presence of cytoplasmic droplets of neutral lipids may significantly affect the P values of DPH-labeled cells (37), which would disturb the correct estimation of the fluidity in the cellular membranes. Therefore, we have checked our cell samples for vacuolar lipids by histological staining. Although MO₄ cells cultured with 30 µg/ml indeed showed evidence of some vacuolization, indicative of their bad condition, which might contribute to the extremely decreased P value, there was no evidence for the presence of a difference between lipid droplets in control MO₄ cells or cells grown with 3 or 10 µg of ET-18-OCH₃ (results not shown). The data, therefore, suggest that ET-18-OCH₃ induces a fluidization in MO₄ cellular membranes.
DISCUSSION

In the present in vitro assay, ET-18-OCH₃ was found to inhibit the invasion of MO₄ cells at concentrations that permitted assembly of microtubules, did not affect directional migration, partly inhibited growth, and did not significantly influence viability of MO₄ cells. Analogous results were obtained with another analogue of ALP (ET-18-OH) and with a thioether-lysocephospholipid. It is unlikely that ET-18-OCH₃ inhibited invasion following the same mechanism as microtubule inhibitors which affected invasion, microtubule assembly, directional migration, and growth at approximately the same concentration (18, 21, 23, 33).

Partial inhibition of growth could hardly be held responsible for the effect of ET-18-OCH₃ on invasion, since several agents that completely inhibited growth of MO₄ cells were shown to permit invasion (18, 21, 23, 32). Furthermore, increase of the number of MO₄ cells (larger aggregates) did not alter their behavior in front of the normal tissues when ET-18-OCH₃ was present in the medium.

The molecular mechanism of action of ALP (ET-18-OCH₃, among others) is believed to be as follows (1, 8, 25). ALP is incorporated into cytomembranes and eventually, for example ET-18-OH and others, without substitution of the lyso group in sn-2 degraded by O-alkyl cleavage enzyme. Relative lack of this enzyme might lead to accumulation of ALP, together with the fact that derivatives substituted in sn-2 (for example, ET-18-OCH₃) cannot be reacylated by acyltransferases and are not necessarily suitable substrates for O-alkyl cleavage enzymes. Consequent disturbance of the normal phospholipid metabolism might be partially responsible for the cytostatic and cytotoxic activities of ALP (25). MO₄ cells appeared to be more resistant to these activities of ET-18-OCH₃ than most other malignant cell lines. For example, 5 μg of ET-18-OCH₃ per ml completely blocked proliferation of Meth A mouse sarcoma cells (25). Therefore, it is probable that MO₄ cells have a relatively high O-alkyl cleavage activity, although it is not excluded that the aforementioned disturbance of the phospholipid metabolism is responsible for the anti-invasiveness of ET-18-OCH₃. This leads to the hypothesis that ET-18-OCH₃ interferes with invasion through alterations of the plasma membrane of the MO₄ cells which precede lethal effects. This hypothesis is supported by the overall increase of membrane fluidity in MO₄ cells treated with antiinvasive concentrations of ET-18-OCH₃. Such alterations might affect the expression of glycolipids and glycoproteins that were held responsible for the malignant behavior of cells (28, 30). Whether ET-18-OCH₃ also affects the membrane fluidity of the heart cells and whether this might eventually contribute to its antiinvasive effect are currently investigated.

Inhibition of metastasis might be due to antiinvasiveness or to interference with one or more of the other steps involved in the formation of metastasis (13). The in vivo experiments using the Lewis lung model did not provide arguments in favor of the possibility that the antimetastatic effect of ALP was due to their antiinvasive activity, since in most of these experiments (5, 6, 9), the drugs also exerted their effect on metastasis when administered after amputation of the primary tumor. Obviously, this does not exclude that in other treatment models (5, 26) the antiinvasive activity of ET-18-OCH₃ might contribute to its antitumoral effect.

In conclusion, we consider that ET-18-OCH₃, and possibly other ALPs, were interesting tools to study mechanisms of tumor invasion since, at least for MO₄ cells in vitro, they have pointed to as yet unknown aspects of these mechanisms.

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Fig. 1. Light micrographs of aggregates of MO4 cells explanted on glass without drug (a), with 1 µg (b), 10 µg (c), and 30 µg (d) of ET-18-OCH₃ per ml. Fixation 7 days after addition of drug and staining with H & E. *, large polynucleated cells; arrow, focus of proliferating cells. Scale bars, 100 µm. Insets, whole cultures from which details were made; scale bar, 1 mm.

Fig. 2. Light micrographs of MO4 cells seeded on glass, fixed after 3 days and stained with antiserum against tubulin. a, After 2-hr treatment with 1 µg of nocodazole per ml; b, same as a with subsequent washing and further incubation in culture medium without drug for 2 hr; c, same as a with subsequent washing and further incubation for 2 hr in medium containing 30 µg of ET-18-OCH₃ per ml. Scale bars, 20 µm.
Fig. 3. Light micrographs of 8-μm-thick sections from aggregates of MO<sub>4</sub> cells with an original diameter of 0.2 mm (a and b) and of 0.4 mm (c and d) confronted with fragments of cardiac muscle without drug (a and b) and with 10 μg of ET-18-OCH<sub>3</sub> per ml (c and d). Fixation after 4 days; a and c are stained with H & E; b and d, with an antiserum against chick heart. M, MO<sub>4</sub> cells; H, heart. Scale bars, 100 μm.

Fig. 4. Light micrographs of 8-μm-thick sections from aggregates of MO<sub>4</sub> cells (diameter, 0.2 mm) confronted with lung fragments without drug (a) and with 10 μg of ET-18-OCH<sub>3</sub> per ml (b). Fixation after 4 days. Staining with H & E. M, MO<sub>4</sub> cells; L, lung tissue. Scale bars, 100 μm.
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