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Inhibition of induced angiogenesis in a human microvascular endothelial cell line by ET-18-OCH_3

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Abstract. Alkyl-lysophospholipids are a group of anticancer compounds that have previously been shown to have the unique feature of being selectively toxic to neoplastic tissues. One of these compounds, ET-18-OCH_3, has been used for purging bone marrow of cancer cells in phase I clinical trials. Tumor-induced angiogenesis has been directly correlated with tumor growth and metastasis. In this study, we examined the effect ET-18-OCH_3 has on a human microvascular endothelial cell line (HMEC-1), including the following functions: angiogenesis, cell-adhesion molecule expression, and cell-junction integrity. We found that ET-18-OCH_3 (in vitro) reversibly inhibited induced angiogenesis at levels that did not affect viability. At lower concentrations, ET-18-OCH_3 down-regulated the expression of cell-adhesion molecules and affected the integrity of cell-to-cell junctions. This observation demonstrates this versatile family of compounds to have additional targets of action.

Introduction

ET-18-OCH_3 (ET) is an alkyl-lysophospholipid, a relatively new group of anticancer compounds that have the unique feature of having selective cytotoxicity to neoplastic cells [2, 19]. Human trials have involved the in vitro purging of bone marrow of patients with acute leukemia in remission for later use in autologous bone marrow transplantation [28]. ET has also been used intravenously to treat a group of patients with terminal cancer who have achieved partial (50%) regression of their tumors [3]. The patients treated had bronchogenic carcinoma of different histology, bowel carcinoma, hypernephroma, and acute myeloid leukemia (one case) [3]. Other biological activities associated with ET use include macrophage activation by the induction of B-cells to release a pro-macrophage-activating factor [22] and inhibition of the invasiveness of malignant murine MO_4 cells [25, 26].

Since the early 1970s, antiangiogenic therapy has been proposed as a method for restricting tumor growth [5]. Research evidence suggests that in the absence or inhibition of angiogenesis, tumor growth can be severely restricted [5–7, 9, 18]. The concept of angiogenesis inhibition has generated a wide search and interest for new compounds that can inhibit tumor growth and metastasis [8, 10, 20]. Antiangiogenic compounds can be of very different types and include cytokines such alpha-interferon, which has been used in clinical trials to treat hemangiomas in children [29]; minocycline, a semisynthetic tetracycline derivative that has anticollagenase activity [12, 27]; steroids [4, 14]; synthetic heparins [11]; and sulfated chitin derivatives [21, 24].

In this study, we observed three new effects of ET. These effects were observed using the newly described human microvascular endothelial cell line CDC/EU.HMEC-1 (HMEC-1) [1] and included angiogenesis inhibition, a decrease in the expression of adhesion molecules, and a decrease in the ability of monolayers of microvascular endothelial cells to maintain tight junctions.

Materials and methods

Cell culture. HMEC-1 cells were grown to confluence and dispersed with trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Grand Island, N.Y.). The cells were counted and their viabilities were determined by trypan-blue dye-exclusion test. The growth medium consisted of endothelial basal medium (EBM; MCDB 131, Clonetics, San Diego, Calif.), 10 ng epidermal growth factor (EGF)/ml (Collaborative Biomedical Products, Bedford, Mass.), 1 μg hydrocortisone (Sigma, St. Louis, Mo.), and 15% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, Utah).

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Tubule assay. Matrigel (Collaborative Biomedical Products, Catalog number 40234, Bedford, Mass.) was added (200 µl/2 cm^2) to 24-well tissue-culture plates and allowed to gel for 30 min at 37° C. Each well was then seeded with 10^5 HMEC-1 cells, which were allowed to attach for 1 h at 37° C. Thereafter, the volume of each well was adjusted to 1 ml with EBM growth medium. All tubule assays were observed at between 18 and 24 h of incubation at 37° C. We judged that tubule formation was inhibited when over 75% of the cells present in a well did not form connecting tubes between them.

Tubule-inhibition assay. ET-18-OCH_3 (Medmark Chemicals, Grunwald, Germany) was diluted in EBM without serum to a stock solution of 2 µ*M*. Concentrations of 4, 11, 21, 43, 64, 85, and 107 n*M* (final concentration) were used in the assays. One assay involved adding ET during the tubule-formation phase; the other consisted of preincubating HMEC-1 cells with ET for 45 min, 4 h, and 24 h prior to plating on Matrigel. For the preincubation study, ET was added at T_0 or at 24 h after inoculation of 6-well plates with HMEC-1 cells. All tubule-inhibition assays and viabilities were evaluated by the same scorer, performed in duplicate, and repeated at least once. Toxicity of ET against HMEC-1 cells was positive when the viability (as evaluated by a trypan-blue dye exclusion test) of the cells fell below 70% of the control value. The viability of healthy HMEC-1 cells was normally above 95%. We judged that tubule formation was inhibited when more than 75% of the cells in the well did not form connecting tubes between them.

Adhesion-molecule assay. The effect of ET on the expression of selected endothelial adhesion molecules CD29 (AMAK, Inc., Westbrook, Me.), CD31 (AMAK, Inc.), CD44 (Becton-Dickinson, San Jose, Calif.), CD54 (Becton-Dickinson), CD34 (Becton-Dickinson), VCAM-1 (Becton-Dickinson), and CD49d (Becton-Dickinson) was evaluated. HMEC-1 monolayers that were 80%–90% confluent were allowed to incubate for 24 h with 32 n*M* ET (final concentration). Each assay contained 5×10^5 cells/ml that were washed three times in phosphate-buffered saline (PBS, 0.01 *M*; pH 7.40) supplemented with 0.1% bovine serum albumin and 0.1% sodium azide, after which 25 µl of a 1:5 dilution of primary antibody was added to the cell pellets, which were then incubated for 30 min at 4° C. The cells were then washed three times, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody was added, and the cells were incubated for 30 min at 4° C. The results were read using a FACScan reader (Becton-Dickinson).

Cell-junction integrity model. The integrity of HMEC-1 cell junctions in the presence of ET was examined. The assays were run in 24-well plates using Costar (Cambridge, Mass.) 6.5-mm Transwell porous cell-culture inserts. EBM (0.8 ml) was added to the outer well, the insert was placed in the well, and 7×10^4 HMEC-1 cells in 100 µl EBM were added to the insert. The plates were incubated at 37° C in an atmosphere containing 5% CO_2 until the cells formed a confluent monolayer. At this time, ET was added to the insert and the plates were reincubated for various periods. At the end of the incubation period, 5 µl [^{3}H]-thymidine (Dupont, Wilmington, Del.) was added to the insert, samples were removed from the outer well at 10-min intervals, and the number of counts were determined using an LKB liquid scintillation counter (Wallace, Gaithersburg, Md.).

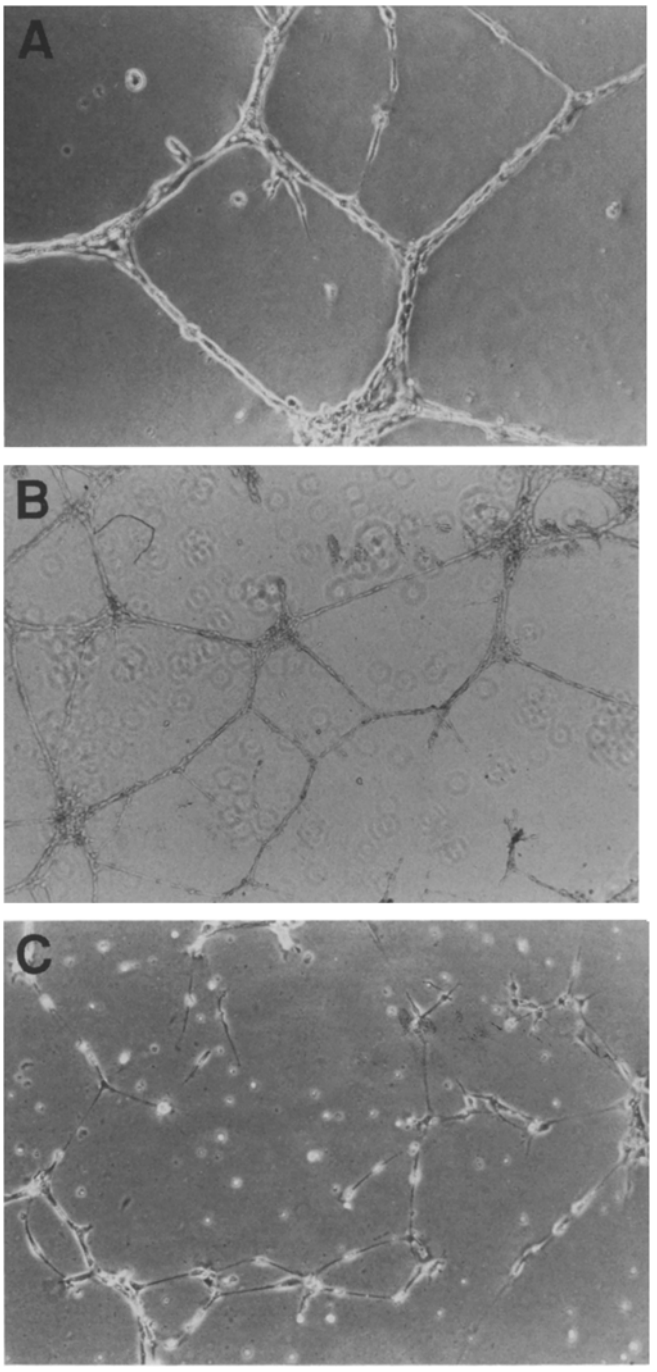

Fig. 1A–C. HMEC1 cells forming tubules in Matrigel after approximately 20 h of incubation at 37° C. **A** Control (without ET, ×200). **B** Control (×100). **C** ET at 64 n*M* (final concentration, ×100)

Results

We observed that when ET was present during the tubule-generation period in Matrigel, inhibition occurred at concentrations of 43 n*M* or higher (Table 1, Figs. 1A–C). Toxicity, judged by viabilities lower than 70%, was not observed at concentrations of 64 n*M* or less in any of the experiments. In addition, when ET was present during the tubule-formation process at inhibitory levels of 64 and 85 n*M* for 24 h and the drug was then removed, tubules formed in the 64-n*M* wells. When the cells were preincubated with ET for 45 min, 4 , and 24 h and then washed, the tubule-inhibition results varied (Table 2). At preincubation times of 45 min and 4 h with ET, we found the effect to be reversible at concentrations of up to 85 n*M*. When the preincubation time was extended to 24 h, tubule formation was inhibited at concentrations of 43 n*M* and above.

FACScan analysis was performed on HMEC-1 cells after incubation with ET. We observed a down-regulation of four endothelial adhesion molecules when ET (32 n*M*) was present: CD54 (ICAM-1), CD44 (HCAM), CD29

Table. 1. Tubule inhibition when ET-18-OCH_3 was continuously present in the medium

	ET-18-OCH_3 dose (n*M*)						
	0	4	11	21	43	64	85
ET with DMSO	–	–	–	+	+	+	ND
ET medium only	–	–	–	–	+	+	+
25% DMSO, 75% EBM without serum	–	–	–	–	–	–	–

+, Greater than 75% inhibition of tubule formation by cells; ND, not determined

Table 2. Tubule-formation inhibition when cells were preincubated with ET-18-OCH_3 before being inoculated onto Matrigel

	ET-18-OCH_3 dose (n*M*)[a]						
	0	4	11	21	43	64	85
45 min	–	–	–	–	–	–	+
4 h	–	–	–	–	–	–	+
24 h	–	–	–	–	+/–[b]	+	+

+, Greater than 75% inhibition of tubule formation by cells

[a] Cells died or had decreased viability at concentrations of 85 n*M* and above

[b] Only 60% inhibition was observed

(integrin beta-1), and CD34. The mean percentages of decrease were: CD54, 65%; CD44, 68%;, CD29, 39%; and CD34, 19%. Other markers showed no resultant increase or decrease, including CD49d, CD31, and VCAM. Cell-junction integrity data were generated to examine whether the decrease in cell-adhesion molecules would result in the cells' becoming less cohesive. Our data demonstrate that after treatment with ET, the monolayer lost the ability to retain its fluid interphase, allowing leakage to occur at a 50%–60% greater rate (Table 3). Untreated cells did not lose their tight association.

Discussion

Tubule formation by endothelial cells in reconstituted basement membrane has been taken to correlate with the angiogenesis process in vivo [13, 16–18]. In an earlier paper [1], we demonstrated the formation of microtubules by HMEC-1 microvascular endothelial cells to be similar to that of primary foreskin microvascular endothelial cells; this was the reason why we chose the HMEC-1 cell line for our in vitro studies.

We clearly demonstrated that ET has antiangiogenic activity in vitro (Table 1, Figs. 1 A, 1 B) and that this effect might be translated to an in vivo model of angiogenesis (currently under study). We observed that ET has the additional advantage of not being toxic to the HMEC-1 cells at levels that inhibit angiogenesis in vitro as demonstrated by their high viability. Viability starts decreasing at dose levels of ET above 85 n*M* (Tables 1, 2).

Whether this inhibition of angiogenesis can be translated to inhibition of tumor growth remains unknown; however, Folkman and other investigators [5–7, 18] have presented strong arguments that tumors are angiogenesis-dependent and that unrestricted growth of tumors is dependent on angiogenesis. Additionally, there is strong evidence that ET itself may be cytotoxic to certain tumor cell types [2, 3, 19, 28], thus lending credibility to the possibility that in a given appropriate delivery system, ET could be an effective antitumorigenic agent when used alone or in combination with a known chemotherapeutic agent.

We found that at 32 n*M*, ET significantly decreased the expression of several known adhesion molecules, including integrin beta-1 (CD29), involved in wound healing, tissue invasion, and tumor metastasis; HCAM (CD44, homing-associated cell-adhesion molecule), which relates to normal- and tumor-cell tissue development as well as to lymphocyte homing; and ICAM-1 (CD54, intercellular adhesion molecule 1), involved in neutrophil transmigration and the inflammatory response process. Since CD29 is directly involved in wound healing, tumor metastasis, and endothelial cell arrangement during angiogenesis, this compound again demonstrated potential practical application in the chemotherapy of solid tumors. How these cell-adhesion molecules are down-regulated remains an enigma; however, it is known that phospholipid synthesis is inhibited and signal transduction is perturbed by alkyl-lysophospholipids such as ET [15, 23]. Using a model that examines cell integrity and mimics the intact vascular bed, we demonstrated the confluence of endothelial monolayers on micropore filters to provide a functional barrier to the passage of radiolabeled material. After treatment with ET at different intervals, we found the cells to allow radioactive material through in a dose-response-related fashion.

Taken together, these data suggest that ET disrupts cell integrity and down-regulates the expression of cell-adhesion molecules, which suggests a lack of the ability to generate microtubule formation, or the inhibition of angiogenesis.

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Table 3. Cell-junction integrity: fluid retention

	10 min	20 min	30 min	40 min	50 min	60 min
Control	549	1,033	1,907	3,488	4,378	7,026
15ul ET (24h/5h)	1,130	2,272	4,341	7,255	10,534	14,082
20ul ET (5h)	838	1,683	3,636	5,359	7,862	10,359

Data are expressed in cpm

[a] Values are statistically significant

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