Removal of Breast Cancer Cells from Bone Marrow by in Vitro Purging with Ether Lipids and Cryopreservation

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ABSTRACT

Four cell lines from human breast cancer (HTB 19, HTB 133, IZB B, and MCF 7M) were investigated for in vitro growth before and after incubation with the ether lipid 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH3) for 4 h and at increasing concentrations (25, 50, 75, and 100 µg/ml), as well as with and without cryopreservation. When measured with the human tumor cloning assay in agar or methylcellulose, the surviving fraction showed an inverse correlation with the concentrations of ET-18-OCH3. After a 4-h exposure with 75 µg/ml ET-18-OCH3, at a cell density of 2 x 10⁶/ml the number of colonies of HTB 19 decreased from 75 ± 10³ cells (100%) to 1 ± 0³ cells (1%), and after subsequent cryopreservation no remaining colonies were found. In order to simulate the situation in autologous bone marrow transplantation we contaminated normal human bone marrow cells with malignant HTB 19 breast cancer cells at a ratio of 100:1. After incubation with 75 µg/ml ET-18-OCH3 for 4 h and subsequent cryopreservation there was a considerable reduction of HTB 19 colonies whereas the majority of normal human hematopoietic progenitors recovered. We conclude that ET-18-OCH3, in combination with cryopreservation, can remove breast cancer cells from bone marrow by up to 1 log and may be useful for purging bone marrow for autologous bone marrow transplantation not only in acute leukemia and non-Hodgkin's lymphoma but also in breast cancer.

INTRODUCTION

High-dose chemotherapy with autologous bone marrow transplantation is a promising approach to the treatment of advanced metastatic and hormone-unresponsive breast cancer [Refs. 1–4; see Antman (1) and Eddy (3) for review]. Autologous bone marrow transplantation-supported high-dose chemotherapy is also under investigation as adjuvant treatment for high risk disease (5). One potential limitation of autologous bone marrow transplantation, however, is the possibility that clonogenic metastatic tumor cells might contaminate the bone marrow which is reinforced following ablative chemotherapy. Nineteen to 32% of women without bone marrow metastases by standard criteria were found to have bone marrow involvement at the time of diagnosis when more sensitive techniques including immunocytochemical and immunohistochemical methods for detection of occult metastases in breast cancer were used (6–12). Although the importance of either overt or occult bone marrow metastasis in autologous bone marrow transplantation of breast cancer is still unknown there is concern that occult clonogenic tumor cells in the infused marrow may contribute to relapse.

Therefore, several interesting approaches to ex vivo removal of breast cancer cells from involved bone marrow have been developed using mainly pharmacological purging and antibody-mediated methods. The former has been conducted most commonly with the active cyclophosphamide derivatives 4-hydroperoxycyclophosphamide or mafosfamide. Antibody-based methods use complement-mediated cytotoxicity or immunomagnetic beads. Other more recently developed methods include the use of the long-term marrow culture system and photodynamic therapy (13–20). Another possible approach for purging bone marrow from breast cancer cells is the use of cytotoxic drugs such as ether lipids (21–25).

Ether lipids were found to have direct cytotoxic effects in a variety of neoplastic tissues. In addition, antitumor activity in vivo can be observed in various mouse models (21). Berger et al. (26) showed some effect in the therapy of autochthonous methylnitrosourea-induced mammary carcinoma in Sprague-Dawley rats. Andreason et al. (27) demonstrated that these compounds had some selectivity toward neoplastic tissues and spared normal cells. Subsequently, it was found in experiments on myelomonocytic leukemia WEHI-3B cells that the ether lipid ET-18-OCH3 clearly showed selective cytotoxic effects (25, 28). The exact mechanism for this selectivity has not yet been firmly established [see the paper of Berdel (21) for review]. Nevertheless, because of its selectivity, clinical phase I/II studies were initiated in patients with acute leukemia and with non-Hodgkin's lymphoma of high malignancy to assess the safety and efficacy of in vitro bone marrow purging with ET-18-OCH3 in setting of autologous bone marrow transplantation (24).

This paper reports in vitro results on ET-18-OCH3 as a putative purging compound for autologous bone marrow transplantation in patients with breast cancer.

MATERIALS AND METHODS

Drugs. The ether-lipid ET-18-OCH3 was purchased from Medmark Chemicals. Its further handling in our experiments has been published in detail previously (22).

Human Breast Cancer Cell Lines. The human breast cancer cell lines HTB 19 and HTB 133 were purchased from the American Type Culture Collection (Rockville, MD). Both the other breast cancer cell lines IZB B and MCF 7M were kindly provided by Dr. M. Schmitt, Technische Universität, Munich, Germany. All cell lines were continuously cultured at 37°C in 5% CO2 humidified atmosphere in culture medium (Eagle's minimal essential medium) containing 10% heat-inactivated FBS. All cultures were prepared in a laminar air-flow hood with sterile technique.

Normal Human Bone Marrow Cells. After informed consent about 10 ml of bone marrow aspirates were collected from the posterior iliac crest with heparinized syringes from donors with histologically normal bone marrow.

In Vitro Incubation with the Drug. Two x 10⁵/ml cells of the four cell lines of human breast cancer (HTB 19, HTB 133, IZB B and MCF 7M) were investigated for in vitro growth before and after incubation with the ether lipid ET-18-OCH3 for 4 h and at increasing concentrations (25, 50, 75, and 100 µg/ml), as well as with and without cryopreservation.

Incubation was performed at 37°C in 5% CO₂ in high humidity as well as in lightly capped disposable plastic culture tubes in tube culture medium (human breast cancer cells) or in RPMI 1640 (normal human bone marrow progenitor cells) containing 10% FBS. The cells were pelleted by centrifugation and resuspended for clonogenicity assays and cryopreservation procedures.
**Human Clonogenic Tumor Assay.** The assay was performed using the capillary tube system first described by Maurer et al. (29) and with the later described modifications (HTCAcap) (30). Two × 10^6 cells/ml were incubated with ET-18-OCH₃, pelleted by centrifugation, resuspended at a cell density of 1 × 10^6 cells/ml, and then divided in two parts. One half of the cells were suspended in culture medium containing 20% FBS and 10% DMSO (Sigma) and cryopreserved at a final concentration of 5 × 10^6 cells/ml. The "capillary incubation mixture" consisted of 300 µl cell suspension (containing the other half of cells (= 5 × 10^6 cells/ml) in double enriched CMRL-1066 medium [for further ingredients and handling see the paper of Berdel et al. (30)]. Aggregates consisting of more than 40 cells were scored as colonies. Decrease in colony formation under the influence of ET-18-OCH₃ was interpreted as cytotoxicity on the level of the self-regenerating capacity of the cells. Standard deviation within triplicate cultures was ± 20%. The numbers of colonies ± SD for the controls are shown in Table 1, and the surviving fraction in the drug-treated and/or cryopreserved capillary samples was calculated as for the normal human hematopoietic progenitors (see below).

**Clonogenic Assay for Hematopoietic Progenitor Cells from Normal Bone Marrow.** The CFU-GEMM assay in methylcellulose was performed according to a method by Fauser and Messner (31) in two very similar and previously described modifications, A and B (22).

The surviving fraction was calculated as follows: The number of hematopoietic progenitors (CFUs) in the control plates was defined as being 100% and expressed as 1.00. The numbers of hematopoietic progenitors (CFUs) in drug-treated and/or cryopreserved plates were expressed as a percentage of the control before cryopreservation × 1.00 ± SD.

**CFU Assay for Remission Marrows Contaminated with Breast Cancer Cells.** Normal bone marrow cells were mixed with human breast cancer cells of the HTB 19 cell line in a ratio of 100:1 and incubated at 37°C in 5% CO₂ for 4 h with 75 µg/ml ET-18-OCH₃. Incubation was performed in a plastic culture tube containing a 1-ml final volume at a final cell concentration of 2 × 10⁶/ml. After the incubation period, the cells were washed twice with a culture medium of the HTB 19 cell line containing 10% FBS and assayed for colony formation (CFU-GEMM, modification B) before and after cryopreservation. The growth of the HTB 19 cell line in this mixed culture could be markedly improved if 3% agar was added to the modification B medium at the same ratio (1:5:5) as in the HTCAcap. HTB 19 colonies were easily differentiated from normal hematopoietic CFUs such as CFU-GEMM, burst-forming unit-erythrocytes, colony-forming unit-erythrocytes, and even colony-forming unit for granulocytes and monocytes morphologically based on description of the colonies in methylcellulose (Fig. 1). Many spot checks were performed by taking single colonies out of the dish preparation for staining procedures which confirmed the diagnosis.

**Cryopreservation and Thawing of Cells.** After incubation with various doses of ET-18-OCH₃, one half of the cells were cryopreserved before testing in the clonogenic assays. After removal of the drug by centrifugation, cells were suspended in culture medium containing 20% FBS and 10% DMSO (Sigma), and a 1-ml volume at a final cell concentration of 2 × 10⁶/ml (in HTCAcap) or 2 × 10⁷/ml (in clonogenic assay modifications A and B) was placed in freezing vials (Nunc, Denmark). The vials were then frozen in Styropro to −90°C overnight and stored in the vapor phase of liquid nitrogen. Rapid thawing of the cells and reduction of concentration of DMSO by dilution in medium as well as by addition of DNase when cell clumping occurred were described previously (22).

**Statistical Methods.** The statistical comparison of the cytotoxic activity of ET-18-OCH₃ and cryopreservation against human breast cancer cell lines with no drug controls before cryopreservation was performed by the two-tailed Friedman test followed by multiple comparisons based on Friedman rank sums (32). The same activity against normal human bone marrow cells was compared by the two-tailed t test. P < 0.05 was considered as indicating significant differences and P < 0.01 was considered as indicating highly significant differences.

### RESULTS

The results show that in each of the four human breast cancer cell lines there is a clear *in vitro* drug dose-response relationship. The cytotoxicity of ET-18-OCH₃ was enhanced by subsequent cryopreservation (Table 1). One example of IZB B is shown in Fig. 3 in addition to Table 1. Thus, for instance after a 4-h exposure with 75 µg/ml ET-18-OCH₃ at a cell density of 2 × 10⁶/ml, the number of colonies of HTB 19 decreased from 75 ± 10/10³ cells (100%) to 1 ± 0/10³ (1%) and after subsequent cryopreservation no colonies recovered. At the next higher dose of 100 µg/ml ET-18-OCH₃ there was no colony formation left either before or after cryopreservation.

In contrast, a 4-h exposure of ET-18-OCH₃ to 75 µg/ml or less of drug did not significantly decrease the colony formation of normal human hematopoietic progenitors (Table 2). Experiments of incubation with 0, 25, and 50 µg/ml ET-18-OCH₃ have been published previously (22). Data on exposure to 75 µg/ml ET-18-OCH₃ were achieved for this publication. Furthermore, there was no significant change in the distribution of the morphologically different colony types after purging (data not shown). Freezing, storing, and thawing yielded a loss of about 45% in the colony survival (Table 2).
Table 2 Effect of ET-18-OCH₃ and cryopreservation on the clonal growth of normal human hematopoietic progenitors after 4 h of drug incubation

Experiments with 50 μg/ml ET-18-OCH₃ and less were performed according to modification A and with 75 μg/ml ET-18-OCH₃ according to modification B of the test (see "Materials and Methods"). The surviving fraction was calculated as outlined in "Materials and Methods," means ± SD.

<table>
<thead>
<tr>
<th>Dose of ET-18-OCH₃ (μg/ml)</th>
<th>Cells/ml</th>
<th>Before cryopreservation [surviving fraction (CFUs/plate)]</th>
<th>After cryopreservation [surviving fraction]</th>
<th>No. of donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5×10⁶</td>
<td>0</td>
<td>0.65 ± 0.17</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>0.63 ± 0.20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.59 ± 0.13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2×10⁷</td>
<td>0</td>
<td>0.64 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>0.55 ± 0.02</td>
<td>3</td>
</tr>
</tbody>
</table>

*p < 0.05, t test (two-tailed) for comparison of means between colony recovery after ET-18-OCH₃ ± cryopreservation treatment and no-drug controls before cryopreservation at the same time of incubation. All other corresponding comparisons yielded no significant differences.

As shown in Table 3, in each of the three experiments with simulated remission bone marrow conducted by contamination with malignant HTB 19 cell lines there was an excessive growth of breast cancer cells if no ET-18-OCH₃ was added. One example is shown in Fig. 2 in addition to Table 3. An incubation of 4 h at 75 μg/ml ET-18-OCH₃, however, reduced the HTB 19 cell population to 14–28% and spared normal progenitor cells (99–121%). Following cryopreservation, the HTB 19 colonies were markedly reduced to 8–9%, corresponding to a 1 log growth reduction of breast cancer cells, whereas the normal hematopoietic progenitor cell lines always showed a recovery of more than 50% (52–77%).

DISCUSSION

These in vitro purging studies indicate that solid tumor cell lines such as human breast cancer cell lines also show some sensitivity to ET-18-OCH₃ as was demonstrated in similar previous in vitro experiments for leukemic as well as lymphoblastic cell lines. ET-18-OCH₃ showed a clear dose-activity relation, and the cytotoxic effects were further augmented by cryopreservation (Table 1; Fig. 3).

The toxic effect of the ether lipid ET-18-OCH₃ seems to be greater on breast cancer cells than on normal human bone marrow progenitor cells. These preclinical studies revealed that incubation of a mixture of...
Breast cancer cells and normal human bone marrow cells with ET-18-OCH₃ led to a dose-dependent antitumor effect without impairing the ability of bone marrow cells to reconstitute. Therefore, ET-18-OCH₃ offers the possibility of preferentially removing breast cancer cells while retaining adequate numbers of normal hematopoietic cells for subsequent infusion into the patient in the setting of autologous bone marrow transplantation.

In addition, these results are in accordance with former in vitro studies undertaken by Vogler et al. (25), in which similar remission could be achieved with a dose of 100 μg/ml ET-18-OCH₃, since in former studies (22) the maximum purging effect was always achieved after 4 h and not after comparing times of 1 or 18 h of drug exposure.

Experiments of incubation with the higher dose of 100 μg/ml ET-18-OCH₃ were not conducted for several reasons. In previous works clumping was a frequent technical problem making cell counts more and more difficult. In addition, the higher dose of 100 μg/ml ET-18-OCH₃ proved to be too cytotoxic to bone marrow progenitor cells. Whereas 75 μg/ml ET-18-OCH₃ spared more than 50% of bone marrow progenitor cells previous studies have shown that bone marrow progenitor cells were often eliminated or reduced to more than 50% by the higher dose of 100 μg/ml ET-18-OCH₃. Lastly, one of our patients with acute myeloid leukemia received a second transplant comprising marrow purged with 100 μg/ml ET-18-OCH₃. He subsequently failed to engraft (24).

In addition, these results are in accordance with former in vitro studies undertaken by Vogler et al. (25), in which similar remission bone marrow transplantation on leukemic patients it was concluded that incubation at 37°C for 4 h at 75 μg/ml ET-18-OCH₃ is the purging dose recommended for a phase II study (24).

Whereas in our HTCAexp experiments the HTB 19 cell line could be completely eliminated by a 4-h exposure to 75 μg/ml ET-18-OCH₃ and subsequent cryopreservation, in the simulated remission bone marrows, these procedures led only to a partial reduction of the malignant human breast cells, i.e., by 1 log to 8–9%. The cell line HTB 19 which was selected for the mixture experiments proved to be as sensitive as IZB B to a 75-μg/ml drug exposure for 4 h and subsequent cryopreservation and only slightly more sensitive than HTB 133 and MCF 7M (Table 1; Fig. 3). Nevertheless, the results of these ether lipid purging experiments with suspensions of marrow and HTB 19 cells show less purging activity of the drug in breast cancer cells than in leukemic and lymphoblastic cell lines (22).

ET-18-OCH₃ has several properties that make it an attractive candidate for use in chemoseparation of breast cancer cells from human bone marrow. The drug is easy to formulate and requires a short treatment time, an important consideration when using human bone marrow that must be harvested and frozen on the same day.

The molecular basis for the direct and differential cytotoxicity of the ether lipid ET-18-OCH₃ is not yet completely understood (21). It is assumed that interference with normal phospholipid metabolism causes higher concentrations of the drug in neoplastic cells, which may lead to direct destruction of the altered cell. Furthermore, there is some evidence that alkyl-lysophospholipids induce differentiation of neoplastic cells (33).

The clinical relevance of purging is still uncertain (14, 18, 19). Breast cancer may, however, be a disease in which the importance of purging might eventually be tested. These observations provide a rationale for the reduction of neoplastic cells from bone marrow suspensions by ex vivo incubation with ET-18-OCH₃.

From the results of this study it is concluded that ET-18-OCH₃ may be useful as a bone marrow-purging agent not only for patients with acute leukemia and malignant non-Hodgkin’s lymphoma but also for patients with breast cancer in the setting of autologous bone marrow transplantation. Since, however, the purging effect of ET-18-OCH₃ is less pronounced in breast cancer cells than in leukemic and lymphoma cells further studies must be done to enhance the preferential antineoplastic effect of this drug. This might possibly be achieved by additional treatment procedures such as immunoseparation, cytokines, or hyperthermia. On the other hand, the possible impairing impact on growth of the normal human bone marrow progenitor cells must clearly be considered and investigated further (34, 35).
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REFERENCES

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