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Original Paper

Expression of Two 50 kDa Proteins is Associated with Sensitivity Towards Etherlipid Analogues in the Human Leukaemia Cell Line HL 60

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Hexadecylphosphocholine (HePC) is a new etherphospholipid derived substance with pronounced antineoplastic activity. So far the mode of action of this compound has not been resolved. Therefore, we decided to approach this problem by generating HePC resistant sublines of susceptible cells. The human leukaemia cell line HL 60 was successfully adapted to high concentrations of HePC over a period of 14 months. The resistant cell line HL 60 R shows similar functional characteristics as the original HL 60. Both lines can be induced to terminal differentiation into a granulocytic phenotype by DMSO. In this process, normal HL 60 cells also become resistant towards HePC. Determinations of cellular membrane lipid composition did not show significant changes, which would explain the resistance mechanism. Analysis of cellular proteins by 2D-gelelectrophoresis revealed two 50 kDa proteins expressed in HL 60 and differentiated HL 60 cells, which were not expressed in HL 60 R. Reversion of resistance of HL 60 R after prolonged cultivation without HePC led to re-expression of the two proteins, indicating at a possible involvement of these proteins in HePC sensitivity. © **1997 Elsevier Science Ltd.**

Key words: alkylphosphocholines, etherlipids, hexadecylphosphocholine, mechanism of action, resistance to etherlipids

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INTRODUCTION

HEXADECYLPHOSPHOCHOLINE (HePC) represents a new group of antineoplastic agents which was originally derived from cytotoxic etherlysophospholipids. Besides pronounced antiproliferative effects on tumour cells *in vitro* and *in vivo* [1–6], HePC has other remarkable biological effects. In some leukaemic cell lines and in epithelial tumours HePC induces cellular differentiation [7, 8]. Another interesting characteristic is the inhibition of invasive growth of tumour cells in normal chick heart tissue *in vitro* [9].

Attempts to elucidate the mechanism by which HePC and probably other etherlipid analogues exert their biological effects have not produced a satisfactory answer so far. Although different cell membrane functions, such as protein kinase C activity [10–17], membrane phospholipid metabolism [18–24], signal transduction [25–28] and growth factor binding [29–31] seem to be influenced by these compounds, these effects either do not correlate with the antiproliferative action, or final proof of a correlation is missing.

We tried to approach this problem by generating resistant sublines of HePC susceptible cells. This was successfully accomplished with the epithelial carcinoma cell line KB and the leukaemic cell line HL 60. First comparative studies to characterise these new sublines revealed no striking differences to the original cells at the membrane phospholipid level which could give a sufficient explanation for the resistance mechanism [32, 33]. In this study we tried to elucidate the mechanism of resistance towards HePC further by investigating the impact of differentiation in the leukaemic cell line HL 60 and HL 60 R on phenotypic and functional characteristics of the cells. Special interest was focused on the question of whether resistance induction by cellular differentiation [34] and slow adaptation to HePC are two aspects of the same biochemical process or reflect two independent mechanisms by which the cells cope with cytotoxic etherlipids.

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MATERIALS AND METHODS

Cell culture and biological reagents

HL 60 cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). Cell culture material was obtained from Gibco (Glasgow, Scotland). HePC and [^{3}H]-HePC was provided by Asta Medica (Frankfurt/M., Germany). $HePCPN_6$, ET-18-OCH_3 and BM 41.440 were a gift from H. Eibl, Max-Planck Institute for Biophysical Chemistry (Göttingen, Germany). The cholesterol determination kit and phospholipids were obtained from Sigma (Munich, Germany). Silicagel 60 HPTLC plates were obtained from Merck (Darmstadt, Germany) and organic solvents from Baker (Deventer, Holland). Monoclonal antibodies were obtained from Becton and Dickinson (Heidelberg, Germany).

Cell culture

Cells were grown in RPMI 1640 medium supplemented with 10 mM glutamine, 10 000 U/ml penicillin, 120 U/ml streptomycin and 10% fetal calf serum in a 5% CO_2 atmosphere at 37°C and 95% humidity. For experiments, cells were harvested in their exponential growth phase, washed twice in fresh medium and counted in a Neubauer chamber. HL 60 R cells were generated as described elsewhere [33] and were maintained routinely in HePC-free culture medium for 7 days before each experiment. Cell number and viability, as parameters of the cytostatic and cytotoxic effects of HePC, ET-18-OCH_3 and BM 41.440, were tested in 48 h incubations with a range of concentrations of etherlipids, followed by counting of the cells after staining with trypan blue dye. IC_{50} and LD_{50} values were interpolated from the growth curves obtained by this procedure.

Determination of phospholipids, protein and cholesterol

Cellular lipids were extracted from a known number of cells by the method of Bligh and Dyer [35]. Whole cellular phospholipid content was determined by the method of Eibl and Lands [36]. Total cholesterol was assayed by an enzymic colour reaction according to Sale and associates [37]. Phospholipid subclasses were analysed by applying lipid extracts on silicagel 60 HPTLC plates and separating lipids in a solvent system composed of chloroform/methanol/triethylamine/water 30/34/35/8 (by vol.). Plates were dipped in 10% copper sulphate in 8% phosphoric acid and lipids were visualised by charring at 180°C. The amount of single phospholipid classes was determined densitometrically in a Personal Densitometer (Molecular Dynamics). Cellular protein content was quantified according to Peterson's modification of the method of Lowry [38].

Flow cytometry and NBT-reduction

Cells (5×10^5) were suspended in 100 µl phosphate-buffered saline (PBS) supplemented with 0.01% sodium azide and 2% FCS and incubated for 45 min at 4°C with different fluorescein- or phycoerythrin-labelled mouse antihuman monoclonal antibodies. Cells were washed three times in fresh PBS and cell-associated fluorescence activity was determined in a FACS-scan flow cytometer (Becton and Dickinson, Heidelberg, Germany).

As controls for unspecific binding of each antibody, corresponding isotypic fluorescein- or phycoerythrin-labelled mouse antibodies were employed. NBT reduction was performed by a 1:1 dilution of the cells in culture medium with 0.1% NBT solution and incubation at 37°C for 30 min with 50 nM TPA. The amount of formazan-containing cells was determined in a Neubaur chamber.

2D-gelelectrophoresis

Cell pellets were disrupted mechanically and suspended in buffer (9.5 M urea, 2% nonidet P40, 2% ampholine at pH 3.5–10). The cytosolic and membranous fraction were separated by ultracentrifugation at 100 000*g* for 60 min. Protein material from both fractions was resolved in the first dimension in a 4% acrylamide/bisacrylamide gel. The second-dimension resolution was then performed by SDS–PAGE for 5 h at 40 mA. Gels were stained in 0.5% Coomassie brillant blue in 10% acetic acid and 25% methanol.

[^{3}H]-*HePC uptake*

Cells were incubated with [^{3}H]-HePC 10 µg/ml (10 µCi/µmol) in culture medium at 37°C for indicated time intervals. Aliquots of 5×10^6 cells were taken and washed three times in ice-cold PBS with 10% FCS. Cell pellets were then extracted according to Bligh and Dyer and the radioactivity was determined in the organic phase by liquid scintillation counting.

RESULTS

Induction of differentiation in HL 60 and HL 60 R cells

The expression of differentiation-associated changes of membrane antigens was tested on HL 60 and HL 60 R cells before and after treatment with 1.3% DMSO for 5 days (Table 1). As can be seen, HL 60 and HL 60 R cells showed very similar antigen expression patterns, indicating that induction of resistance by continuous exposure to HePC did not lead to cellular differentiation. On the contrary, both cell variants could be induced to terminal differentiation by DMSO demonstrated by high expression of CD 11c and CD 13 and decreased expression of HLA-DR. Both cell variants

Table 1. Flow cytometry analysis of cell surface antigen expression and NBT-reduction

(%)	HL 60	HL 60 R	HL 60 differentiated	HL 60 R differentiated
CD 11c	19 ± 4*	25 ± 4*	99 ± 1*	98 ± 1*
CD 13	38 ± 3*	16 ± 4*	99 ± 1*	99 ± 1*
CD 15	92 ± 1*	95 ± 2*	83 ± 2*	71 ± 1*
CD 33	95 ± 2	92 ± 1	99 ± 1	99 ± 1
HLA-DR	8 ± 1*	12 ± 1*	1 ± 1*	1 ± 1*
NBT	4 ± 2*	5 ± 2*	78 ± 9*	81 ± 7*

5×10^6 cells were incubated for 45 min at 4°C with monoclonal mouse antihuman antibodies directed at haematopoietic cell membrane antigens as indicated. Cells were washed three times and cell associated fluorescence was quantified in a fluorescence activated flow cytometer. NBT reduction was performed at 37°C for 30 min in the presence of TPA and formazan positive cells were counted in a Neubauer chamber. Data are means ± S.D. of three independent tests. *Significant difference between normal and differentiated cells by Student's *t*-test ($P < 0.05$).

also gained some functional properties of mature granulocytes shown by the strongly increased ability to reduce NBT to formazan.

Cytotoxic effects of etherlipid analogues

In order to see how the differentiated cells react to other etherlipids, we compared the antiproliferative effects of four different etherlipid analogues on HL 60 and HL 60 R cells and their DMSO-differentiated counterparts. Cell viability was determined after 48 h of incubation. The doses required to induce a half-maximal cell kill (LD_{50}) were calculated from dose–response curves (Table 2). HL 60 R cells were approximately 10-fold more resistant to HePC than normal HL 60. Induction of resistance to ET-18-OCH_3 and BM 41.440 could also be observed, although the effect was not as pronounced as for HePC. As expected, $HePCPN_6$ showed minimal cytotoxic activity. Differentiation of normal HL 60 cells by DMSO also induced a 4-fold higher tolerance to HePC and a 2-fold higher tolerance to the other two compounds. Differentiation of HL 60 R cells did not increase the resistance to etherlipids.

Analysis of cellular protein, phospholipids and cholesterol

To test whether cell differentiation leads to changes of basic cellular parameters which would hint at a potential mechanism of resistance, we determined total cellular protein, phospholipids and cholesterol in immature and differentiated HL 60 and HL 60 R cells (Table 3). This analysis revealed only small differences between the cells. The differentiated cells showed less protein, most probably reflecting the reduction in size during differentiation. A slight reduction of phospholipid content was observed, but this change was not significant. Neither the cholesterol content nor the phospholipid to cholesterol ratio showed any correlation with the cellular maturation state or their susceptibility towards HePC. The analysis of single phospholipids by TLC showed only an almost significant reduction of cellular phosphatidylcholine content of approximately 19–22% in HL 60 R and differentiated HL 60.

2D-gelectrophoresis of cellular protein

Since the analysis of cellular lipids did not give a decisive clue for the observed differences of susceptibility to HePC, we also analysed the distribution pattern of cellular protein by 2D-gelelectrophoresis of cell extracts. Interestingly there were two 50 kDa proteins expressed in the cytosolic fraction of HL 60 cells (Figure 1(a)) that were missing in the HL 60 R line (Figure 1(b)). The correlation of these proteins with the resistant phenotype was proven in HL 60 R cells that were cultured without HePC exposure for several months (data not shown). In these cells a parallel loss of HePC resistance and re-expression of the two proteins was observed. In differentiated HL 60 cells (Figure 1(c)), the two 50 kDa proteins were still detectable. Additionally, several other newly expressed proteins were visible.

Uptake of [^{3}H]-HePC

A potential further mechanism of resistance may be a different capacity of HePC uptake in the cellular membranes, therefore, we determined the incorporation of radiolabelled HePC in mature and immature HL 60 and HL 60 R cells over a period of 2 h (Figure 2). HePC uptake was decreased in the differentiated HL 60 cells by 49%. In the HL 60 R cell line, HePC uptake was only 11% of HL 60's level, and differentiation produced hardly any additional decrease.

DISCUSSION

The leukaemic cell line HL 60 can be made resistant to HePC and other etherlipid analogues by slow adaptation [33] or differentiation [34]. We characterised and compared both modes of resistance induction in order to gain more insight into the molecular mechanisms of HePC action.

In HL 60 R cells, resistance was not induced by generation of a more differentiated phenotype during adaptation to HePC. The cells kept their ability to mature into granulocytic forms as demonstrated by immunophenotypic analysis of cell membrane antigens and the ability to reduce NBT. As was expected, DMSO-differentiated HL 60 cells also showed a considerable degree of resistance towards other etherlipid

Table 2. Antiproliferative effects of etherlipid analogues and HePC

(μg/ml)	HL 60	HL 60 R	HL 60 differentiated	HL 60 R differentiated
HePC	2.6 ± 0.6*	25.2 ± 1.8*	11.5 ± 0.6*	24.8 ± 2.0*
ET-18-OCH_3	1.2 ± 0.2*	7.6 ± 0.5*	3.2 ± 0.7*	8.1 ± 0.7*
BM 41.440	1.5 ± 0.2*	6.9 ± 0.4*	3.0 ± 0.4*	7.2 ± 0.3*
$HePCPN_6$	31.5 ± 1.7	> 32	> 32	> 32

2×10^6 cells were incubated with escalating concentrations of test substances for 48 h at 37°C. Cell viability was determined in a Neubauer chamber with trypan blue dye exclusion. LC_{50} values were calculated from dose–response curves and represent the mean ± S.D. of five different experiments. *Significant difference between normal and differentiated cells by Student's *t*-test ($P > 0.001$).

Table 3. Analysis of cell membrane lipids and cellular protein content

10^6 cells	Protein (μg)	Lipid phosphorus (nmol)	Cholesterol (μg)
HL 60	118.0 ± 18.7	27.6 ± 4.1	1.7 ± 1.1
HL 60 differentiated	90.1 ± 11.8*	24.3 ± 6.0	2.2 ± 1.5
HL 60 R	121.9 ± 24.1	30.4 ± 3.5	2.2 ± 0.9
HL 60 R differentiated	92.0 ± 3.0**	26.0 ± 5.4	2.2 ± 1.2

Membrane lipids of 5–10 × 10^6 cells were extracted as described in Materials and Methods. Phospholipids were quantified by lipid phosphorus determination. Cholesterol was measured by an enzymic colour reaction. Proteins were determined by the method of Peterson as described above. Data are means ± S.D. of at least five independent determinations. *significant difference compared with HL60 cells, Student's *t*-test $P < 0.05$; ** significant difference compared with HL60R cells, Student's *t*-test, $P < 0.5$.

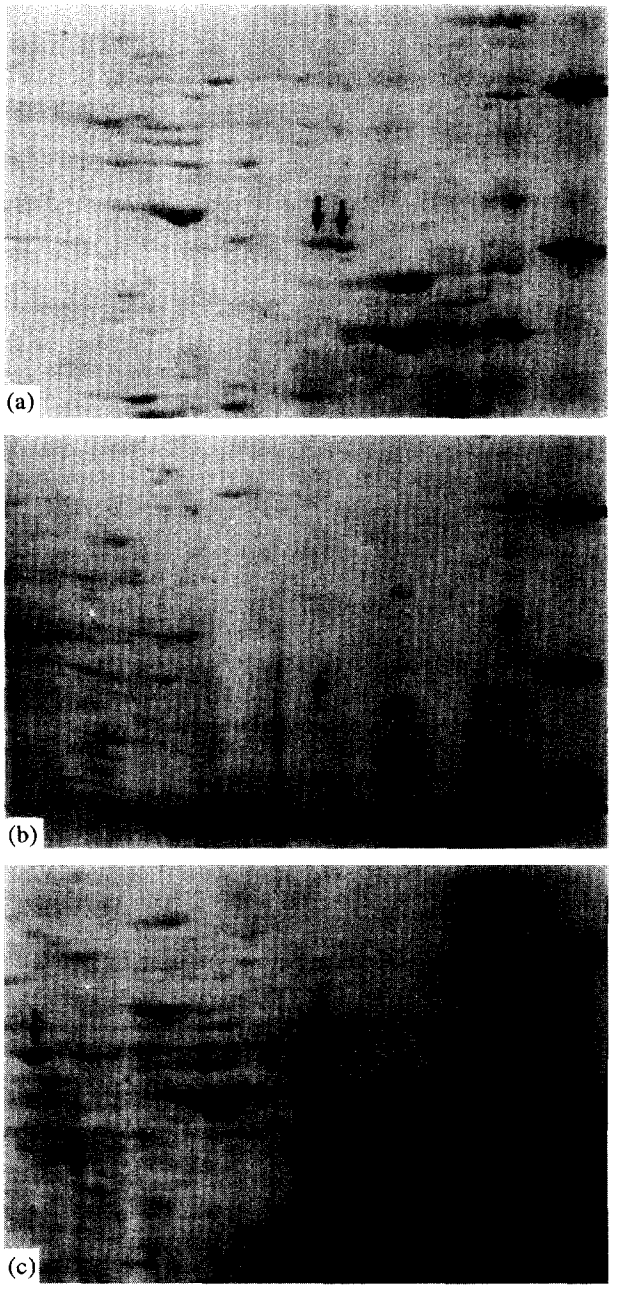

Figure 1. 2D-gelelectrophoresis of cellular proteins. Cellular proteins were separated in a 4% acrylamide gel for the first dimension and then by SDS-PAGE for the second dimension. Proteins from HL 60 cells (a), HL 60 R (b) and HL 60 differentiated (c) were visualised by Coomassie brilliant blue. Consistent differences in protein expression patterns are marked with arrows. Large arrows on the right side mark the positions of the 50 and 81 kDa standards.

analogues, although this was not as pronounced as in the HL 60 R cells. Differentiation of HL 60 R did not produce a higher degree of resistance in these cells. These findings indicate that etherlipids seem to share, to some extent, common modes of cytotoxic action since there is a considerable degree of cross-resistance in differentiated HL 60, HL 60 R and differentiated HL 60 R cells. The different level of resistance between adapted and differentiated cells, and the fact that there is no further improvement of resistance in HL 60 R cells by differentiation, indicates two different mechanisms of resistance induction by adaptation and differentiation.

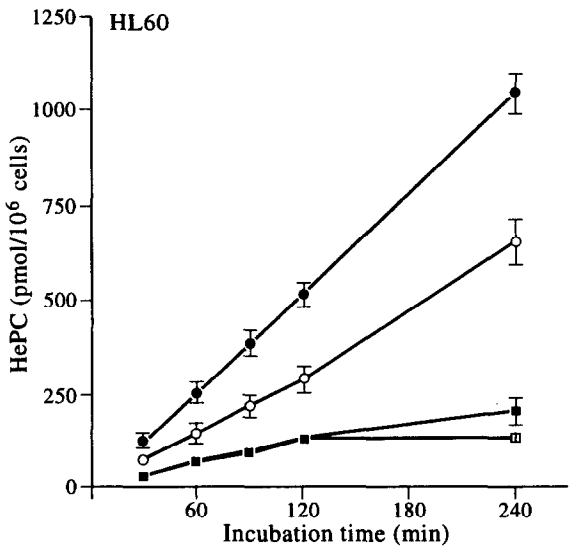

Figure 2. [^{3}H]-HePC uptake. 4×10^6 cells were incubated with 10 μg/ml [^{3}H]-HePC at 37°C for indicated time periods. Cellular HePC uptake was calculated on the basis of incorporated radioactivity determined by liquid scintillation counting. Data are means ± S.D. values of three independent experiments. HL 60 (●), HL 60 differentiated (○), HL 60 R (□) and HL 60 R differentiated (■).

A very suggestive explanation for the observed resistance pattern of the cells was presented by the HePC-uptake experiments, showing a good correlation between cellular sensitivity to HePC and HePC incorporation. It has previously been postulated that resistance to etherlipids is determined by the cellular uptake capacity for these compounds [39] and this may well be a significant resistance mechanism in differentiated cells. In previous investigations, however, we showed that, in spite of having faster uptake kinetics, HL 60 cells, but also the carcinoma cell line KB, did not accumulate higher membrane concentrations of HePC than their resistant counterparts. On the contrary, KB R cells had 3.3 times higher internal HePC membrane concentrations after two days of exposure than KB, while HL 60 and HL 60 R showed identical HePC membrane concentrations. At these levels, the original cell lines already showed impaired viability, whereas in the resistant sublines there were no detrimental effects on cell proliferation [32, 33]. Furthermore, comparison of HePC uptake in various haematological and epithelial cell lines revealed no clear correlation between the sensitivity of the cells and HePC uptake [40].

Other major biochemical parameters of membrane composition between the cells were not greatly different. Although it has been shown that the cellular cholesterol content or the cholesterol to phospholipid ratio in the membrane may be of some importance for etherlipid resistance [41], our results again do not support this hypothesis. Interestingly, a reduction in phosphatidylcholine (PC) content was also found in HL 60 R and differentiated HL 60. It was presumed that reduced PC levels could result from inhibition of PC synthesis by etherlipids via CDP-choline [22]; our observations indicate that a downregulation of PC content may at least in part reflect a yet unidentified mechanism of resistance.

When analysing the expression of cellular protein pattern by 2D-gelelectrophoresis, we detected two cytosolic proteins with a calculated molecular weight of 50 kDa and isoelectric points of 7.4 and 7.6, which were consistently expressed in

HL 60 cells and were always missing in HL 60 R. The significance of the expression of these proteins for the HePC-sensitive phenotype was strengthened by similar findings in KB and KB R cells, and by the observation that HL 60 R cells, when cultured without HePC for more than 6 months, become susceptible to etherlipids again, paralleled by a re-expression of these proteins (data not shown). In addition, in differentiated HL 60 and HL 60 R cells, several new proteins were induced consistently by cell maturation, but the two sensitivity-associated 50 kDa proteins were only expressed in differentiated HL 60.

In our opinion, it seems conceivable that resistance to HePC and other etherlipid analogues may be determined by several independent mechanisms. A downregulation of etherlipid uptake may play a role in some circumstances such as differentiation and may perhaps explain why normal mature tissue such as bone marrow cells or fibroblasts are usually not affected by etherlipid toxicity. Changes of the intrinsic content of etherlipids, cholesterol or PC in the cellular membrane may be another way by which cells could cope with a higher input of external etherlipids to some degree [42]. The third resistance mode, closely linked to the expression of the two 50 kDa proteins, however, seems to be by far the most potent mechanism of cell protection against the detrimental effects of etherlipids on cellular proliferation.

Thus far it has not been proven that any of the proposed molecular modes of action of etherlipids really correlate with the antiproliferative effects of these substances. For the first time, we present evidence of significant structural changes in the protein composition of the cells, directly correlated with sensitivity towards etherlipids. Resistance towards etherlipids and the mode of their antiproliferative action may be closely interlinked. The identification and characterisation of the two sensitivity-associated proteins may therefore hopefully throw some light on the still hypothetical decisive cytotoxic mechanism of etherlipid action. First attempts to sequence and characterise the two sensitivity-associated proteins are currently underway. In the near future, it will be of interest to identify these sensitivity-associated proteins in cells susceptible to etherlipids and to find a lack of expression in naturally resistant cell types.

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