Membrane Vesicle Formation Due to Acquired Mitoxantrone Resistance in Human Gastric Carcinoma Cell Line EPG85–257

Manfred Dietel,1 Hartmut Arps, Hermann Lage, and Axel Niendorf


ABSTRACT

A newly established gastric carcinoma cell line (EPG85–257P) exhibited a high sensitivity to mitoxantrone (DHAD) as determined by a monolayer proliferation assay. The concentration to inhibit cell growth to 50% of controls (IC50) was 0.0022 μg/ml culture medium. The cells were continuously incubated for more than 4 months in the presence of stepwise increased concentrations of DHAD, and the IC50 was increased to 0.41 μg/ml, i.e., 186.4-fold. This resistant variant was named EPG85–257RNO'. The EPG85–257RNO' cells became cross-resistant to Adriamycin with enhanced IC50 by 10.5-fold and to daunomycin with enhanced IC50 by 3.9-fold. No distinct resistance was observed to vinblastine, vincristine, and colchicine. Verapamil (10−8, 4 x 10−8 and 10−7 M) and cyclosporin A (10−7, 1 x 10−7, and 10−6 M) did not reverse DHAD resistance. As shown by immunocytochemistry (monoclonal antibodies: C219 and JSB-1) and Northern blot analysis, DHAD resistance was not associated with the appearance of the multidrug resistance (MDR)-associated (M, 170,000) P-glycoprotein or the overexpression of P-glycoprotein mRNA. The data indicate a chemoresistance pattern unlike typical MDR (often called "atypical" MDR).

The phenotypes of parent and resistant EPG85–257 cells were compared using interference contrast microscopy, electron microscopy, and immunocytochemistry. After DHAD application the following structural characteristics were found to be associated with emergence of resistance: (a) intensive formation of surface vesicles in the resistant variant. Such vesicles were almost absent in sensitive cells; (b) the vesicles contained the selecting DHAD which was visualized by its blue color; and (c) in electron microscopy the vesicles were formed by an inner and an outer double membrane, presumably derived from the plasmalemma. These observations suggest a complex cellular mechanism responsible for DHAD resistance which includes formation of membrane vesicles, vesicular drug binding, and drug compartmentalization.

INTRODUCTION

One limitation of cancer chemotherapy is the emergence of chemoresistant cell populations in malignant tumors. The mechanisms which enable tumor cells to survive and proliferate in the presence of relatively high concentrations of toxic substances are not fully understood. A great number of biochemical studies have described differences between parent (sensitive) cells and the resistant progeny, i.e., altered enzyme activity (e.g., 7-dihydrofolate reductase, glutathione-S-transferase, protein kinase C, topoisomerase II) (1–5), reduced intracellular drug toxification (6, 7), stimulated DNA repair (8–10), mdr gene amplification (11–18), overproduction of binding and transport proteins (e.g., M, 170,000 P-glycoprotein, M, 19,000 protein) (19–22) combined with reduced net drug accumulation (23–25), and increased energy-dependent drug efflux (26, 27). Morphological studies of phenotypic changes due to acquired chemoresistance are relatively rare (28, 29). The experiments of Sehested et al. (30, 31) suggest that enhanced membrane traffic and vesicle formation play a key role in the emergence of drug resistance.

The present study seeks to determine whether resistance-associated phenotypic alterations of cellular and subcellular structures exist. This could be expressed, for example, by an altered membrane exchange, increased vesicular compartmentalization of drugs with augmented extrusion, or altered equipment of subcellular organelles (e.g., lysosomes). To investigate these possibilities we used the human gastric carcinoma cell line EPG85–257 (established in our laboratory), which was initially DHAD sensitive and became resistant to the drug by continuous incubation in the presence of stepwise increased concentrations. The phenotypes of sensitive (parent) and resistant cells were compared by interference contrast microscopy, immunocytochemistry, and electron microscopy, supplemented by Northern blot analysis.

MATERIALS AND METHODS

Human Gastric Carcinoma Cell Line EPG85–257. A surgically removed human gastric carcinoma was delivered to our laboratory in October 1985. The tumor was diagnosed histologically as adenocarcinoma of intestinal type. The procedure for establishing primary cell cultures is described elsewhere (32, 33). In brief, during transport the freshly explanted specimen was kept at room temperature in serum-free medium. The tumor tissue was dissected mechanically by scraping and subsequently digested enzymatically for approximately 15–20 min with 0.1 unit collagenase/ml and 0.8 unit dispase/ml (Boehringer, Mannheim, FRG). After centrifugation (600 rpm at 25°C) the sediment was seeded in culture flasks (Falcon, Heidelberg, FRG). The culture medium was Leibovitz L 15 (Boehringer, Mannheim, FRG) supplemented per 500 ml with 5% fetal calf serum (Biochrom, Berlin, FRG), 500 μg glutamine, 3.1 mg fetuin, 40 IE insulin, 1.25 mg transferrin, 0.56 g NaHCO3, 5 ml minimal essential medium vitamins, and 250 mg glucose (Sigma, Munich, FRG).

After 2 weeks the cells were grown to confluence. To ensure that the cells in culture were derived from the carcinomatous human tumor they were characterized (a) by phase contrast microscopy showing atypical mitoses and dome formation, (b) by immunocytochemistry (see below) demonstrating keratin filaments as proof of epithelial origin (Fig. 14), and (c) by scanning cytophotometry (Leitz MPV 2) measuring nuclear DNA (33, 34). This disclosed 40% atypical cells with DNA values >4.5 n, indicating malignancy of the cultured cells (Fig. 18).

Drugs. The following cytostatic agents were used: DHAD (1,4-dihydroxy-5,8-bis-[12-(2-hydroxyethyl)-amino]ethylamino]-9,10-anthracenedione dihydrochloride; Cyanamid/Lederle, Wolfratshausen, FRG); Adriamycin and daunomycin (Farmitalia, Freiburg, FRG); colchicine (Sigma, Munich, FRG); actinomycin D (MSD Sharp & Dohme, Munich, FRG); VP-16 and cisplatin (Bristol-Myers, Neu-Isenburg, FRG).

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2To whom requests for reprints should be addressed, at Institut für Pathologie, Christian-Albrechts-Universität, Michaelisstr. 11, D-2300 Kiel, Federal Republic of Germany.

1Cyclosporin A (10−8, 10−7, and 10−6 M) did not reverse DHAD resistance.

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Fig. 1. Phenotypic characteristics of the human gastric carcinoma cell line EPG85-257. A, immunocytochemical visualization of the cytoskeletal protein keratin using the antibody KLI indicative of epithelial origin (× 250). B, DNA histogram with a highly atypical pattern of nDNA content proving malignancy of the cultured cells. DNA values (rel. units) are expressed in relation to cultured non-neoplastic fibroblasts (Fig. 2C. arrow).

Table 1 Changes in the IC50 between parent and resistant cells of the human gastric carcinoma cell line EPG85-257

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50* in µg/ml culture medium</th>
<th>EPG85-257P (parent)</th>
<th>EPG85-257RNOV (resistant)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoxantron</td>
<td>0.0022*</td>
<td>0.41^</td>
<td>186.4</td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.001*</td>
<td>0.0105^</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>Daunomycin</td>
<td>0.013^</td>
<td>0.0507^</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.0025</td>
<td>0.0037</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.00005</td>
<td>0.00006</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.00004</td>
<td>0.00004</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.021</td>
<td>0.025</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.002</td>
<td>0.0024</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>VP-16</td>
<td>0.014</td>
<td>0.018</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

* Determined as described in Fig. 2.
^ Significant differences, P < 0.001.

FRG); and vincristine and vinblastine (Lilly, Giessen, FRG). Verapamil was obtained from Sigma (Munich, FRG); cyclosporin A was a gift of Dr. Ryffel (Institute of Pathology, University of Basel and Sandoz Corp., Basel, Switzerland).

Selection of Resistance. The selecting drug was DHAD applied in stepwise increased concentrations for more than 120 days. The initial concentration was 0.001 µm/ml. After the cells were exposed to a particular concentration of DHAD the cultures were allowed to grow to near confluence. Subsequently, they were passaged and exposed to the same concentration for another 2 days, followed by a 2-fold enhancement of the concentration until they grew to near confluence again. During selection control cultures were kept without drug application. At the beginning and the end of this procedure parent and resistant cells were cloned twice by establishing cultures from single cells according to the method described previously (24). Thus, cloned cell lines (parent = EPG85-257P and DHAD resistant = EPG85-
In order to check C219 and JSB-1 specificity and applicability in immunocytochemistry, histological sections of a human adrenal gland known to contain considerable amounts of P-glycoprotein (mdr-1) mRNA were used. The mabs were obtained from V. Ling that had been multiprimelabeled to specific activity (2.5 x 10^6 cpm/mg DNA) with [32P]dCTP (42). Hybridization was done at 42°C in 50% formamide-5x SSC-5x Denhardt’s solution-50 mM sodium phosphate (pH 7.0)-15 mM EDTA-0.1% SDS-250 mg/ml denatured salmon sperm DNA. After hybridization for 36 h the membranes were washed 3 times for 10 min with 2x SSC-0.1% SDS at room temperature followed by washing with 0.1% SSC-0.1% SDS at 66°C for 1 h and subsequent autoradiography.

Sizes of RNA species were determined relative to the positions of 28S and 18S rRNA. As controls CHO-CHC5 cells known to contain high amounts of P-glycoprotein (mdr-1) mRNA were used.

**RESULTS**

Acquired DHAD Resistance of the Human Gastric Carcinoma Cell Line EPG85–257. In the parent cells EPG85–257P, the IC50 of DHAD was 0.0022 μg/ml (Fig. 2). During the 120-day DHAD exposure period the IC50 was enhanced to 0.41 μg/ml in the resistant variant EPG85–257NOV, i.e., by a factor of 186.4 (Table 1). The DHAD resistance remained preserved for at least 90 days when cells were cultured without drug exposure.

Cross-resistance. Experiments on cross-resistance showed an increase of resistance to Adriamycin (10.5-fold) and daunomycin (3.9-fold). However, EPG85–257NOV cells remained fully sensitive to colchicine, actinomycin D, VP-16, cisplatin, vindesine, and vincristine (Table 1).
during the 120 days of parallel incubation.

Reversibility of DHAD Resistance. Addition of verapamil alone for 4 days at concentrations ranging from $10^{-6}$ to $10^{-3}$ M did not influence cell growth significantly, while concentrations higher than $10^{-5}$ M inhibited proliferation rate in a dose-dependent manner. In the noninhibiting range verapamil did not reverse DHAD (0.2 µg/ml) resistance of EPG85–257NOV cells.

Similar results were found with cyclosporin A when added at concentrations of $10^{-6}$, $3 \times 10^{-6}$, and $10^{-5}$ M.

Light Microscopy. If parent and resistant cells were incubated without drug addition, no morphological differences in size, shape, nuclear structure, etc. were observed. However, the application of 1.0 µg/ml DHAD for 24 h induced considerable changes in the phenotype of parent versus resistant EPG85–257 cells. When stained with hematoxylin-eosin, the parent cells exhibited condensed and clotted nuclei as well as shrinkage and lysis of the cytoplasm (Fig. 3A), whereas the resistant cells appeared unaltered (Fig. 3B). In unstained cell preparations of the parent clones an intense blue staining of the nuclei was observed due to accumulation of the dark blue DHAD (Fig. 4A). In contrast, the resistant cells showed completely unstained cell bodies and nuclei, which were both free of the DHAD-specific blue staining (Fig. 4B). It is noteworthy that the resistant cells formed cell surface-associated vesicles which contained DHAD, again shown by the blue staining (Fig. 4, B and C). The vesicles appeared to be of variable size.

Ultrastructure. After 24 h incubation in 1.0 µg/ml DHAD almost all cells of the parent population showed the signs of cell death, i.e., a dark condensed cytoplasm, enlarged mitochondria, a swollen endoplasmic reticulum, and partially disintegrated cell membranes (Fig. 5A).

As seen by light microscopy, the application of 1.0 µg/ml DHAD for 24 h to the resistant variant induced no changes in the cytoplasm, nuclei, or organelles (Fig. 5B) as compared with the untreated cells. However, the most important characteristic in the DHAD-incubated DHAD-resistant cells was the production of multiple vesicles (Fig. 5, B and C). The vesicles were preferentially located near the outer cell surface and appeared to be expelled from the cells. They were formed by an inner and outer surface exhibiting a characteristic eccentric shape. Ultrastructurally, the layers resembled the cell membrane from which they obviously were derived. The membranous layers were separated from each other by an electron-lucent homogeneous material similar to the organelle-free cytoplasm. In this “vesiculoplasma” near the pole of the vesicles a condensed area was often present (Fig. 5C). Intracellular vesicles were observed only rarely.

Immunocytochemistry and Northern Blot Analysis. Neither the sensitive EPG85–257P cells nor the DHAD-resistant EPG85–257RNOV cells exhibited P-glycoprotein immunoreactivity or contained P-glycoprotein-specific mRNA (Fig. 6). This finding was observed with and without drug exposure.

DISCUSSION

In the present study we report a cell line from a human gastric carcinoma (EPG85–257) which developed in vitro chemoresist-
Membrane Vesicle Formation and DHAD Resistance

Fig. 5. Electron microscopy of EPG85-257 cells. Cultured cells after a 24-h incubation in 1.0 μg/ml DHAD. A, parent DHAD-sensitive EPG85-257P cells. The cytoplasm appears rather dark with multiple swollen mitochondria and dilated granules. The plasmalemma shows several breaks with discontinuity of the membrane layers due to cytolysis (primary magnification, × 4400; bar, 0.23 μm). B, DHAD-resistant EPG85-257RNOV cells. The cytoplasm, organelles, and the plasmalemma are well preserved. As demonstrated by light microscopy, multiple vesicles are attached to the surface, presumably derived from the plasmalemma. The resistance-associated vesicles are seen almost only in the resistant variant (primary magnification, × 4500; bar, 0.23 μm). C, vesicles exhibiting an outer and an inner membrane obviously derived from the plasmalemma. The membranes are separated by a homogeneous matrix similar to the organelle-free cytoplasm (primary magnification, × 85000; bar, 0.012 μm).

Resistance for DHAD. We focus on phenotypic changes associated with the in vitro acquired resistance to the selecting drug DHAD. Resistance was induced by culturing the cells in stepwise increased concentrations of DHAD, which resulted in an 186.4-fold increase in IC50. Moderate cross-resistance was found to the related anthracyclines, Adriamycin (10.5-fold) and daunomycin (3.9-fold), but not to other naturally occurring cytostatic drugs, such as actinomycin D, VP-16, vincristine, vinblastine, and colchicine, or cisplatin. Furthermore, verapamil and cyclosporin A in various concentrations did not reverse DHAD resistance, as seen in a number of MDR cell lines. P-glycoprotein or the related mRNA could not be detected in EPG85-257RNOV using immunocytochemistry and Northern blot analysis. The data indicate that a typical MDR pattern (23, 43-48) was not induced. A similar observation has been reported for a leukemic cell line (49, 50), indicating that this incomplete or “atypical” (49) form of MDR is not a unique finding. Recently, another DHAD-resistant leukemic cell line (HL-60/MX) and a colon carcinoma cell line (WiDr/R) were described (51-53) which, like the cell line EPG85-257RNOV, lack cross-resistance to Vincz alkaloids.

With DHAD application the phenotypic differences between parent and resistant EPG85-257 cells were substantiated by disturbed cellular integrity of the parent and unaltered morphology of the resistant variant. Furthermore, the resistant cells created DHAD-containing vesicles. The intensive vesicle formation and release was observed at the apical region of the resistant cells, indicating an outward transport mechanism. The vesicles were derived from the plasmalemma, as shown by electron microscopy. It is noteworthy that these resistance-associated vesicles contained the selecting drug DHAD, as evidenced by the dark blue staining of DHAD. Formation of drug-containing vesicles appeared to represent a defense mechanism by which the resistant EPG85-257RNOV cells rapidly enclose DHAD and thus prevent intracellular drug accumulation. Previously, Cornwell et al. (48) demonstrated biochemically the binding of vinblastine to membrane vesicles of MDR-resistant cells. This indicates that a vesicle-associated drug transport is not a process exclusive to the EPG85-257RNOV cell line but may represent a general way of drug compartmentalization and extrusion. Accordingly, Sehested et al. (30) suggested a vesicular transport of anthracyclines in resistant Ehrlich ascites tumor cells. The vesicle-linked drug efflux appeared to function highly effectively in the resistant EPG85-
MEMBRANE VESICLE FORMATION AND DHAD RESISTANCE

Fig. 6. Northern blot analysis using the pCHP1 probe (0.6-kilobase insert (41)). The autoradiograph demonstrates lack of hybridization with total cellular RNA of EPG85–257RNOV cells (lanes 4 and 6). As control, the highly drug-resistant CHO-CH*C9 cells were used and show a clear hybridization signal (lanes 2 and 7). The parental EPG85–257 cells are shown in lanes 3 and 5, as expected without hybridization signal. Lane I, molecular weight marker I from Boehringer.

257RNOV cells since cytoplasm and nuclei remained almost completely free of DHAD. Under identical conditions nuclei of parent cells stained dark blue by DHAD, cytoplasmic structures disintegrated almost completely, and vesicles did not appear.

From the reported data a working hypothesis concerning the mechanism of DHAD resistance is proposed for the cell line investigated: When the drug approaches the resistant cell it is linked to a binding or receptor protein not present in the sensitive cells. The drug-receptor complex may induce vesicle formation as known for other receptor-mediated processes (54–56). The complex is concentrated near the cell membrane and is rapidly released by exocytosis. This was demonstrated for the first time in the present study, since resistance-associated, drug-loaded vesicles were predominantly located at the surface membrane outside the cell.

The following consideration gives further support: Several investigations, the present one included, report a decreased cellular drug accumulation and an increased drug efflux in resistant variants as compared to parent cells (23, 28, 57). With only one exception (30), none of these studies examined, or only speculated, what happens with the toxic drug when it is released. If resistant cells use a receptor protein to bind the drug and to pump it out of the cell without detoxification, the drug will attack the cell again. Thus, the resistant cell should at least in part inactivate the drug prior to release. One possible way to perform inactivation is to compartmentalize the drug in special vesicles, as shown in the present study. Whether the vesicles are provided with lysosomal activity, as described for intracellular vesicles in the MDR KB-C4 cells (28), remains to be shown.

It has to be clarified whether the described mechanism can generally be attributed to cells resistant to naturally occurring antiproliferating substances. There are hints suggesting a ubiquitous mechanism: (a) cells often exhibit resistance for several biochemically and functionally unrelated drugs (MDR), which requires a general defense mechanism, and (b) several authors described an increase of vesicle formation, drug trapping, and membrane traffic in resistant cells of different origin (30, 31, 47, 48, 58), again supporting the hypothesis that vesicular drug binding, processing, and extrusion might be a general mechanism involved in chemoresistance.

With regard to the in vivo development of drug-resistant tumors, it is still unclear whether the resistant cell clone is one of the heterogeneous cell clones present in each tumor (59) or whether these cells appear de novo, being genetically transformed under the selection pressure. In the present study the parent cell culture, and presumably also the primary tumor, contained a small number of resistant cells as disclosed by their intact morphology following primary drug exposure. This observation, together with several reports (for review see Refs. 5 and 59), suggests that tumors may enclose resistant cells when they reach a clinically significant state. With chemotherapy the resistant mutants are selected by killing the sensitive population. More knowledge is required concerning defense mechanisms of the individual tumor cell and the distribution of resistant versus sensitive cells in a tumor to overcome drug resistance and to give chemotherapy a more permanent effectiveness.

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