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Original Paper

Co-expression of MDR-associated Markers, Including P-170, MRP and LRP and Cytoskeletal Proteins, in Three Resistant Variants of the Human Ovarian Carcinoma Cell Line, OAW42

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Variants of the human ovarian carcinoma cell line, OAW42, exhibiting low-level intrinsic resistance (OAW42-SR) and drug-induced higher-level resistance (OAW42-A1 & OAW42-A), were studied along with a sensitive clonal population (OAW42-S) which was isolated from OAW42-SR. Expression of the MDR-associated protein P-170, the more recently discovered LRP (lung resistance-related protein) and MRP (multidrug resistance-associated protein), topoisomerase IIα and β, GSTπ and the cytoskeletal proteins, cytokeratin 8 and vimentin, were studied (using immunocytochemistry and Western blotting techniques) in conjunction with drug (doxorubicin) accumulation and subcellular distribution. Expression of mRNA for P-170, MRP, topoisomerase 11α and β and GSTπ was studied using RT-PCR (reverse transcriptase polymerase chain reaction). Results indicate differential co-expression of four MDR-associated parameters (P-170, MRP, LRP and reduced topoisomerase IIα and β) in the OAW42-SR and OAW42-A1 variants, whereas resistance in the OAW42-A variant appeared to be mainly P-170 mediated. Comparable amounts of MRP and greater amounts of LRP were detected in the OAW42-S cells compared to the OAW42-SR variant (which showed increased resistance compared to the OAW42-S cells), but all cell lines expressed similar low-level amounts of MRP mRNA (by RT-PCR). GSTπ levels did not differ markedly between variants. Increased levels of the cytoskeletal proteins were observed with increasing levels of resistance. The relative resistance of the variants, OAW42-SR and OAW42-A1, compared with OAW42-S was seen to change during increased serial passaging of the cells. There was greater drug accumulation by the sensitive OAW42-S cell line compared with that of the resistant variants, particularly the most highly resistant OAW42-A cells. Both verapamil and cyclosporin A effectively restored the accumulation defects seen in the resistant variants, cyclosporin A being the more effective of the two. Subcellular location of drug was predominantly in the nucleus with maximum levels seen in the sensitive OAW42-S variant and minimum levels in the most resistant OAW42-A clone. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: P-170, LRP, MRP, topoisomerase IIα, GSTπ, vimentin, cytokeratin, doxorubicin

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INTRODUCTION

TREATMENT OF ovarian carcinoma with a range of chemotherapeutic agents often results in short remission periods, and subsequent relapses are associated with resist-

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ance to chemotherapy. Tumour cells may change, following exposure to chemotherapeutic agents, and exhibit multiple drug resistance (MDR), i.e. become resistant to a broad but clearly defined range of structurally unrelated compounds including anthracyclines, vinca alkaloids and epipodophyllotoxins but not to alkylating agents or β analogues. These changes are often accompanied by alteration of the expression of certain cellular proteins, e.g. P-glycoprotein, (PgP), multidrug resistance-associated protein (MRP), lung resistance-related protein (LRP), GSTπ or topoisomerases. Physiological changes affecting the structure of the plasma membrane, inter- and extracellular pH and rates of drug transport in and out of the cell are also implicated in resistance [1]. Ovarian carcinomas are known to have significant inter- and intratumoral antigen heterogeneity [2], and it has been postulated that, following initial treatment with chemotherapeutic drugs, intrinsically more resistant, minor cell populations within the mixed tumour mass become the dominant population [3]. Paired sensitive and resistant variants of cell lines established from tumours appear to be useful experimental models for the study of different modes of resistance and may lead to more efficient chemotherapeutic/circumvention regimes [4].

MATERIALS AND METHODS

Cell lines

The OAW42 cell line, originally derived from the ascites of a patient with serous cystadenocarcinoma of the ovary [5], was obtained from the ECACC (European Collection of Animal Cell Cultures), Porton Down, U.K. Four variants of this cell line are described. OAW42-SR is a heterogeneous cell population which we derived by serial subculture of OAW42 and which exhibits resistance to doxorubicin (relative to the drug-sensitive OAW42-S) without exposure to the drug *in vitro*. For this study, OAW42-SR cells were studied between passages 80 and 102. The OAW42-S cell line is a drug-sensitive clonal subpopulation isolated in this laboratory from OAW42-SR and studied between passages 1–20 postcloning. Resistant variants, OAW42-A1 and OAW42-A (both uncloned, heterogeneous cell populations) were derived from OAW42-SR by prolonged exposure to doxorubicin at 0.4 μg/ml and 0.75 μg/ml, respectively. For this study, OAW42-A1 and OAW42-A were studied between passages 74 and 82 and passages 94 and 102, respectively. All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS), L-glutamine (2 mM), 0.1% sodium pyruvate, 7.5% $NaHCO_3$ and Hepes (1 M) at 37°C. Antibiotics were not used in the growth media of the above cell lines. Positive and negative control cells for LRP detection i.e. the non-small cell lung carcinoma resistant cell line, SW-1573/2R120, and its sensitive counterpart, SW-1573, were cultured in DMEM supplemented with 5% FCS, L-glutamine (2 mM), $NaHCO_3$ and Hepes (1 M) as above. These cells were supplied by Professor R.J. Scheper, Free University Hospital, Amsterdam, The Netherlands.

Positive and negative control cells for MRP detection i.e. the promyelocytic leukaemia cell line, HL60ADR, and its sensitive counterpart, HL60, were cultured in RPMI 1640 supplemented with 10% FCS and L-glutamine (2 mM) in a humidified atmosphere of 5% CO_2 at 37°C. These cells were supplied by Dr Melvin Center, Kansas State University, Manhattan, Kansas, U.S.A.

Antibodies

The *MDR-1* gene product P-170 was detected by immunocytochemistry and Western blotting with murine antibodies JSB-1 (Serotec, U.K.), C219 (Centocor, France) and 6/1C (anti-P-170 specific antibody developed in this laboratory). LRP (P-110) was detected by immunocytochemistry only using murine LRP-56 antibody supplied by Professor R.J. Scheper. MRP was investigated by immunocytochemistry and Western blotting using both a rat monoclonal antibody, MRPr1, obtained as a gift from Professor R.J. Scheper and a rabbit polyclonal antibody received as a gift from Dr Melvin Center. Topo 11α and β were studied using monoclonal antibodies to each, supplied as a gift from Dr G. Astaldi-Ricotti, Istituto del Consiglio Nationale del Ricerche, Pavia, Italy, and an anti-Topo 11α monoclonal antibody, clone 4/2D, raised in this laboratory. GSTπ was detected by immunoblotting and immunocytochemistry with the polyclonal rabbit anti-GSTπ antibody, A3600, from DAKO Ltd, U.K. Cytokeratin 8 and vimentin were detected by indirect immunofluorescence with anti-cytokeratin 8 (M631 from DAKO Ltd, U.K.) and anti-vimentin antibody (V5255 from Sigma ImmunoChemicals, U.K.).

Cytotoxicity assays

Toxicity to doxorubicin and a range of other drugs was determined by the acid phosphatase method [6]. Briefly, cells were seeded in 96-well microtitre plates (1×10^4 cells/ml) and incubated at 37°C overnight. Antibiotics were not used in the culture medium. Cells were then exposed to various concentrations of drug for 5 days. At the end of this incubation period, cell survival was determined by addition of the acid phosphatase substrate, *p*-nitrophenyl phosphate. Cytotoxicity tests were carried out at least in triplicate (i.e. 3×96-well plates) and each drug concentration replicated in eight wells of the 96-well plate (i.e. each drug concentration replicated at least 24 times) and the mean $\pm$ standard deviation calculated.

Western blotting

Western blotting for the detection of P-170 was performed using either plasma membrane enriched microsomal fractions [7] or crude whole cell lysates of the OAW42 variants. Crude cell lysates were used for the detection of MRP and GSTπ. Protein concentrations of cell membranes/lysates were determined by the BCA protein assay [8]. Prior to Western blotting [9], cell proteins were separated on SDS-polyacrylamide gels [10]. Various detection methods were employed for detection of protein on blots, i.e. when using alkaline phosphatase-linked secondary antibody, the substrate system 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) was used; horseradish peroxidase-linked secondary antibodies were detected with 3-amino-9-ethylcarbazole (AEC) or the enhanced chemiluminescence (ECL) technique, which has considerably increased sensitivity over the other detection methods.

Immunocytochemistry

P-170, LRP and MRP were detected on cytospins of the OAW42 variants using the ABC method [11]. A Strep-ABC/HRP-conjugated kit plus appropriate biotinylated secondary antibodies were obtained from DAKO Ltd. Briefly, cytospins were made on APES-coated slides (3-aminopropyltriethoxysilane, obtained from Sigma) and left to air dry

overnight at room temperature. Slides were then wrapped in tinfoil and stored at −20°C until required. Before use, slides were brought to room temperature for at least 15–20 min. For the detection of P-170 using C219, 6/1C and JSB-1, cells were fixed for 2 min, 1 min and 10 min, respectively, in ice-cold acetone. For the detection of LRP, MRP (both monoclonal and polyclonal antibodies) and GSTπ, cells were fixed for 10 min in ice-cold acetone. All slides were then air-dried for at least 15 min prior to immunostaining. Endogenous peroxidase activity was blocked by placing slides in 0.6% (v/v) H_2O_2 in methanol for 5 min at room temperature. All cells were blocked with 20% normal rabbit or goat serum (depending on species in which secondary antibody was raised) for 20 min at room temperature. Primary antibodies appropriately diluted in TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) were applied for 2 h at room temperature, followed by a 30 min incubation with the appropriate biotinylated secondary antibody diluted in TBS (rabbit anti-mouse IgG 1/300, rabbit anti-rat IgG 1/100 and goat anti-rabbit IgG 1/500). Finally, the StrepABComplex/HRP was applied for 25 min. Slides were washed between each incubation in three changes of TBS/0.05% (v/v) Tween 20 within 15 min. The horseradish peroxidase substrate, DAB (3,3-diaminobenzidine tetrahydrochloride) containing 0.02% H_2O_2 was applied for 10–15 min at room temperature. Cells were then lightly counterstained with Coles's haematoxylin for 3–4 min, differentiated in 1% acid alcohol and blued in Scott's tap water. After dehydrating in graded alcohols, the cells were cleared in xylene and glass mounted with DPX (BDH, U.K.).

Detection of cytokeratin 8 and vimentin was by indirect immunofluorescence studies. Cytospins were prepared and stored as above. Prior to immunostaining, cells were fixed for 2 min in ice-cold acetone and allowed to air-dry for 20–30 min. Primary antibody was applied for 1 h at 4°C in a humid container. Following three washes with PBS (Dulbecco A, 10 mM, pH 7.3), FITC-labelled (1/50 dilution in PBS) secondary antibody (Boehringer Mannheim, Germany) was applied for 1 h at 4°C in a humid container. Following three washes with PBS, slides were mounted with Vectashield (Vector Laboratories, U.K.) mounting medium and viewed using a Nikon phase-contrast microscope fitted with an FITC filter. In all immunocytochemical studies, appropriate negative control slides were included using irrelevant ascites, supernatant or diluent alone.

Measurement of intracellular doxorubicin accumulation

Intracellular accumulation of drug was measured by a modification of the spectrofluorimetric assay of Ganapathi and Grabowski [12]. Briefly, variants of the OAW42 cells were plated in six-well plates at $2–5 \times 10^5$ cells/well and incubated for 48 h at 37°C. The growth medium was then aspirated and replaced by fresh medium containing doxorubicin (Farmitalia, U.K.) at 10 μM in the presence or absence of verapamil (Sigma, U.K.) at 30 μM or cyclosporin A (Sandoz, U.K.) at 10 μM. At specified time points, the drug-containing medium was removed and the cells rinsed twice in ice-cold PBS. The doxorubicin was directly extracted with a 0.3 N HCl/50% methanol solution and measured using a Perkin-Elmer LC50 luminescence spectrophotometer at an excitation wavelength of 470 nm and an emission wavelength of 585 nm. Cells from replicate wells were trypsinised and counted using a haemocytometer in order that results for accumulation could be expressed as drug content per cell.

Intracellular doxorubicin distribution

Studies on the subcellular distribution of drug were performed by the method of Chauffert and associates [13]. Cells were plated on to sterile coverslips in Petri dishes and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. After exposure to doxorubicin (10 μM) in the presence or absence of verapamil (30 μM) or cyclosporin (10 μM), the coverslips were rinsed in ice-cold PBS, inverted on to slides and sealed with silicone grease to protect against dehydration. The intracellular localisation was studied by ultraviolet illumination, which incudes an orange fluorescence at the site of doxorubicin accumulation.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from all cell lines using standard methods [14] and quantified spectrophotometrically at 260 nm and 280 nm [15].

All reagents used in the reverse transcription and polymerase chain reactions were from Promega (U.S.A.) except where otherwise stated. cDNA was synthesised from 1 μg of total cellular RNA in 20 μl of a solution containing 1 μg of oligo$(dT)_{12-18}$ primers, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM each dNTP, 40 U RNasin and 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, U.K.). The reverse transcription (RT) reaction was incubated at 37°C for 1 h followed by incubation at 95°C for 2 min [14]. The cDNA was either used directly or stored at −20°C until required.

Typical PCR reactions were set up as 50 μl volumes using 5 μl of cDNA from the RT reaction in a solution containing 10 mM Tris-HCl, pH 9.0, 5 mM KCl, 0.1% Triton, 1 mM $MgCl_2$, 200 μM each dNTP, 250 ng of first-strand target primer, 250 ng of second-strand target primer, 125 ng of first-strand endogenous control primer, 125 ng of second-strand endogenous control primer and 2.5 U *Taq* DNA polymerase enzyme. The reaction mixture, prior to the addition of *Taq* enzyme and dNTPs, was denatured initially at 94°C for 5 min to prevent non-specific binding of primers to cDNA. On the addition of dNTPs and *Taq* enzyme to each reaction tube, the cDNA was amplified by PCR (Techne, PHC-3) using the following programme: (1) denaturation of double-stranded cDNA at 95°C for 1.5 min; (2) amplification in the exponential phase (mdr-1, MRP, Topo 11, Topo 11α for 25 cycles: GSTπ for 20 cycles; Topo 11β for 27 cycles) at 95°C for 1.5 min, 54°C for 1 min (annealing of primers) and 72°C for 3 min; and (3) extension at 72°C for 7 min. β-actin was used as the endogenous control for all reactions. Primer sequences have been published previously [14]. The products formed were analysed by agarose gel electrophoresis and densitometry (Imaging Densitometer, Bio-Rad, U.K. model GS-670). Duplicate RNA extractions and duplicate PCR reactions for each extraction were performed and results concurred.

RESULTS

Cytotoxicity profiles of OAW42 variants

The responses of the different variants (at the passage level indicated) to doxorubicin, vincristine and VP-16 are shown in Table 1. The relative resistance of the variants

Table 1. Typical resuls obtained for cytotoxicity assays on OAW42 variants (at the passage number specified) with 3 different drugs are shown. Results shown are the mean ± standard deviation of eight replicates done in triplicate for each drug tested. The standard dviation in all cases was less than 10%

Drug IC_{50} (nM)	OAW42-S Passage 20	OAW42-SR Passage 101	OAW42-A1 Passage 80	OAW42-A Passage 98
Doxorubicin	53.3 ± 6.2	441 ± 33.0	749 ± 54.2	1380 ± 429
Vincristine	15.1 ± 1.3	304 ± 16.7	212.8 ± 19.7	608 ± 51
VF-16	914.9 ± 77	1921.3 ± 128	1829 ± 102	6861 ± 421

Table 2. Relative resistance (at passage number specified) of OAW42-SR, OAW42-A1 and OAW42-A variants compared with the sensitive OAW42-S cells

Drug	OAW42-SR Passage 101	OAW42-A1 Passage 80	OAW42-A Passage 98
Doxorubicin	8.3	14.0	25.8
Vincristine	20.0	13.8	40.0
VP-16	2.1	2.0	7.5

compared with the sensitive OAW42-S cells at the passages indicated is shown in Table 2.

There appeared to be an upward drift in the resistance level with increasing passage number in the case of the OAW42-SR and the OAW42-A1 variants (data not shown). The OAW42-SR variant, for example, showed an increase in doxorubicin IC_{50} from an average of 152 nM at passage 80 to an average of 441 nM at passage 101 (i.e. relative resistance compared with OAW42-S cells ranging from 3–8-fold); and for the OAW42-A1 variant, the corresponding change was from an average 375 nM at passage 74 to an average of 749 nM at passage 80 (i.e. relative resistance compared with OAW42-S cells ranging from 8–14-fold). In contrast, no changes in resistance levels were noted for OAW42-S cells postcloning (passages 1–20), and no significant changes in IC_{50} values were observed for OAW42-A cells (passages 94–102) (which were approximately 26–27-fold more resistant than OAW42-S; data not shown). In these cytotoxicity assays, standard deviations from the mean were calculated to be less than 10% for all drug concentrations monitored (each drug concentration was replicated at least 24 times).

*P*170/mdr-1 *detection*

All three methods (Western blotting, immunocytochemistry and RT-PCR), employed for the detection of *MDR-1* mRNA and protein in the variants, produced concordant

Table 3. Shows typical densitometry results (volume analysis) for OAW42 variants from Western blots. Three separate experiments were performed for each of the antigens examined and blots were analysed by densitometry with similar results obtained for each analysis

Cell line	P-170	Topo IIα	Topo IIβ
OAW42-S	0.00	2.31	4.78
OAW42-SR	0.09	0.38	2.34
OAW42-A1	0.35	0.25	1.90
OAW42-A	3.45	2.59	4.44

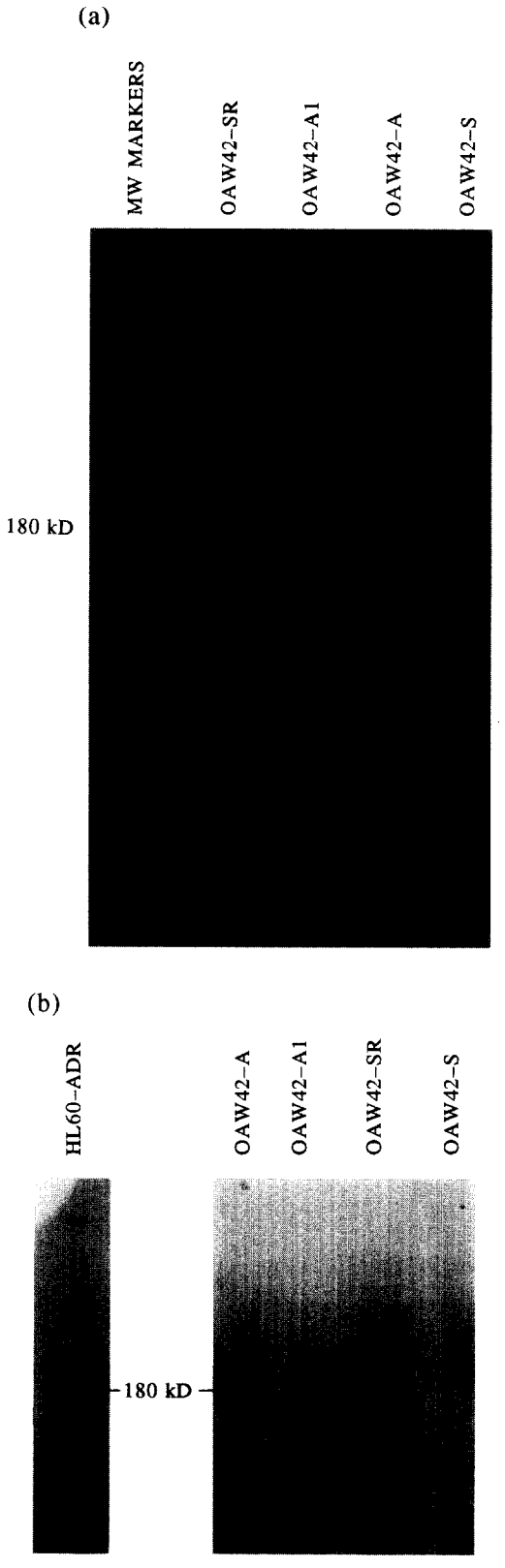

Figure 1. Crude cell lysates (15 μg protein) of OAW42 variants probed for (a) P-170 with antibody C219 (1 μg/ml) detected using alkaline phosphatase-linked rabbit anti-mouse secondary antibody and BCIP/NBT substrate system. (b) MRP with antibody MRPr1 (1/40 dilution) detected using horseradish peroxidase-linked rabbit anti-rat secondary antibody and enhanced chemiluminescence (ECL); 3 min exposure time.

results. With all methods, *MDR-1* was shown to be significantly overexpressed at the mRNA and protein level in the OAW42-A variant; overexpressed, but to a lesser extent, in the OAW42-A1 cells; and expressed, but at a relatively low level, in the OAW42-SR variant. No *MDR-1* mRNA or P-170 were detected in the OAW42-S sensitive cells. Typical Western blot and agarose gel electrophoresis results of RT-PCR products for the variants are shown in Figure 1a and Figure 2a, respectively. Densitometry results are shown in Table 3. Although immunocytochemical results concurred with the overall expression pattern of *MDR-1* observed, some increase in the total number of cells stained and staining intensity within cell populations was seen with increasing passaging of OAW42-SR cells and especially in OAW42-A1 cells (data not shown). This was not noticeable with the OAW42-A cells where the majority of cells showed very positive staining at all passage levels examined.

MRP detection

All OAW42 variants were examined for the presence of MRP by Western blotting, immunocytochemistry and RT-PCR. Two separate antibodies were used for immunodetection, a rabbit polyclonal antibody and a rat monoclonal antibody, MRPr1. Results using both antibodies for immunocytochemistry were inconclusive as 'sticky', apparently non-specific binding was seen on the variants. The MRP-positive control cells showed good positive staining with both antibodies. Results obtained for Western blotting studies depended on the detection method used. When the BCIP-NBT substrate was used to detect alkaline phosphatase-linked secondary antibody or AEC substrate was used for the detection of horseradish peroxidase-linked secondary antibody, all the variants appeared negative for MRP (only the positive control cells showed positive staining). However, when the more sensitive ECL method of detection was used (secondary antibody linked to horseradish peroxidase) all variants showed positivity for MRP with antibody MRPr1 as shown in Figure 1b. OAW42-S and OAW42-SR variants appeared to have comparable amounts of MRP, both having increased amounts of MRP over the OAW42-A1 or OAW42-A variants. Although comparable amounts of MRP were detected in OAW42-S and OAW42-SR cells, the latter cells were shown to be approximately 3–8-fold more resistant (depending on passage number studied) than the OAW42-S cells. RT-PCR studies indicated that mRNA for MRP was present (at a very low level) in all the OAW42 cell lines (Figure 2b).

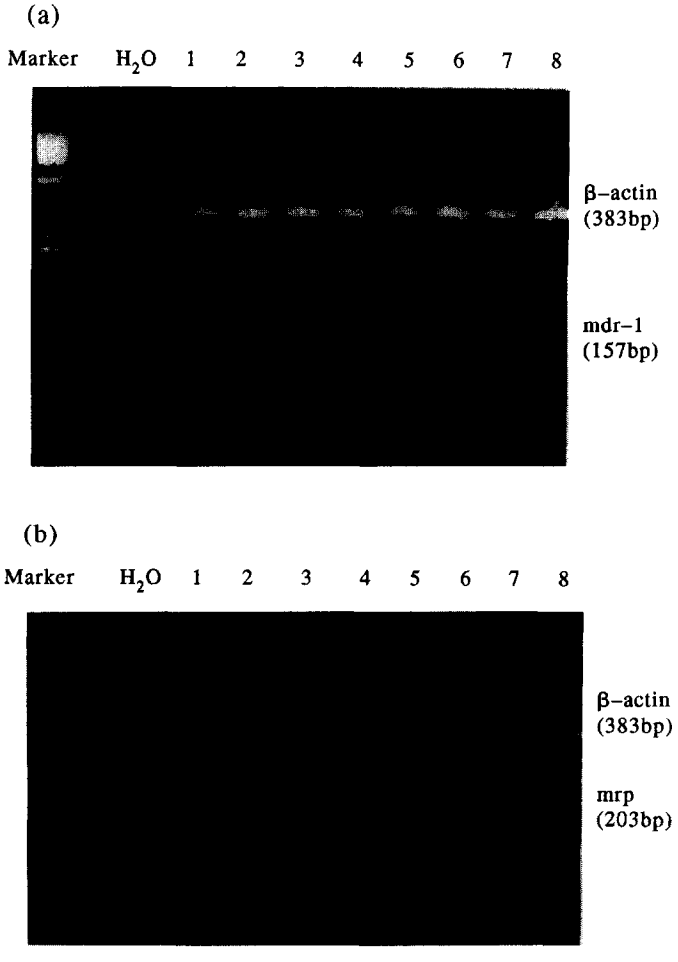

Figure 2. Agarose gel electrophoresis of RT-PCR product for the OAW42 variants. (a) *MDR-1* mRNA; (b) *MRP* mRNA. Lanes 1 and 2 show results for OAW42-A cells; lanes 3 and 4 show results for OAW42-A7 cells; lanes 5 and 6 show results for OAW42-SR cells; and lanes 7 and 8 show results for OAW42-S cells.

LRP detection

The LRP-56 antibody was used for the detection of this antigen by immunocytochemistry. Immunoprecipitation studies by Scheper and associates [16] have identified an antigen of 110 kDa recognised by this antibody. Very early passages of the OAW42-S cells showed no staining for LRP, but upon increasing passaging, staining was seen on all cells. However, the cytotoxicity profile of these latter passages did not differ significantly from earlier passages (data not shown), suggesting that the LRP expressed may be functionally inactive in terms of conferring drug resistance. Significant differences in the percentage of cells stained by LRP-56 was noted between lower (P86, approximately 10–15% cells stained) and higher (P92, approximately 80% of cells stained) passages of OAW42-SR (Figure 3). These differences in percentage cell staining occurred over 6–8 passages of the OAW42-SR cells. However, unlike the OAW42-S cells, an increased level of resistance (monitored by cytotoxicity assays) was associated with this increase of cell staining (data not shown). Notable differences in staining pattern were not noted between different passages of the OAW42-A1 variant (<20% cells stained) and no LRP was detectable in OAW42-A cells. Results for positive control cells, SW-1573/2R120, confirmed that conditions for LRP staining were correct, giving strong granular outer cyto-

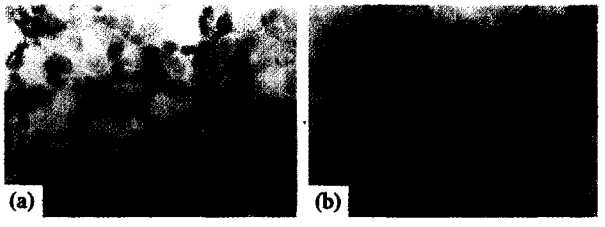

Figure 3. OAW42-SR cells stained for LRP with monoclonal antibody LRP-56. (a) Passage 86, (b) Passage 92 (scale bar = 25 μm).

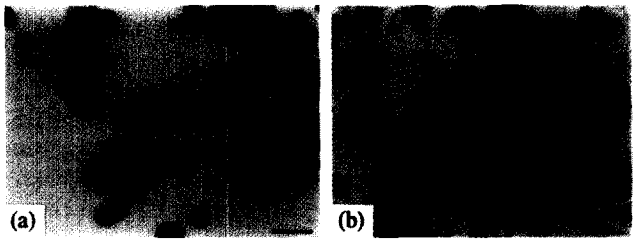

Figure 4. (a) OAW42-S cells (passage 10) and (b) OAW42-A1 cells (passage 80) stained for Topo IIα with monoclonal antibody clone 4/2D (scale bar = 25 μm).

plasmic staining as seen for the positively stained OAW42 variants. Weak LRP staining was observed on the sensitive parental cells, SW-1573.

Topoisomerase IIα and β detection

Western blotting using pooled monoclonal antibodies to Topo IIα and β indicated that levels of both isoforms were reduced in the OAW42-SR and the OAW42-A1 cells compared with OAW42-S cells. However, levels of both isoforms in the OAW42-A cells were comparable to those seen in the OAW42-S cells (Table 3). Immunocytochemical studies using the anti-Topo 11α antibody (clone 4/2D) raised in this laboratory concurred with these results, with a greater percentage of cells staining positive on cytospins of OAW42-S and OAW42-A cells compared to the other two variants (Figure 4). RT-PCR studies were in agreement with these results with the exception of the results for the OAW42-A variant which showed somewhat decreased levels of both isoforms compared to the sensitive OAW42-S variant at the mRNA level (data not shown).

GSTπ detection

When investigated both by immunocytochemistry and Western blotting, levels of GSTπ in all the resistant variants showed little change from the sensitive OAW42-S cells. RT-PCR studies indicated a slight increase of GSTπ at the mRNA level in the OAW42-A cells compared to the two other resistant variants and the sensitive OAW42-S cells (data not shown).

Detection of cytoskeletal proteins, vimentin and cytokeratin 8

Results from the indirect immunofluorescence studies on the variants showed an increase in the number of cells stained and the staining intensity as drug resistance increased, i.e. very faint staining was observed for the sensitive OAW42-S cells, and lower passages (e.g. P86) of the OAW42-SR cells, whereas the OAW42-A cells exhibited intense staining for both cytoskeletal proteins (Figure 5). The staining pattern of the OAW42-SR cells was most interesting as the number and intensity of cells stained increased in the higher cell passages (e.g. P92) showing increased resistance levels and LRP-56 positivity.

Doxorubicin accumulation in the OAW42 variants

Figure 6 illustrates doxorubicin accumulation in each of the four variants over a period of 3 hours. The results demonstrate a linear increase in the amount of doxorubicin accumulated in 3 hours. The results demonstrate a linear increase in the amount of doxorubicin accumulated by the OAW42-S within the time period covered. After 3 hours, the OAW42-S cells had accumulated approximately 600 pmol adriamycin/10^6 cells. Doxorubicin accumulation was significantly reduced in both the OAW42-SR and OAW42-A1 variants compared with the OAW42-S cells, with a maximum cellular concentration of 390 pmol/10^6 cells. The uptake rate and cellular drug concentrations were similar to the OAW42-S cells within the first hour studied; subsequently, a plateau was observed in both the OAW42-SR and OAW42-A1 cells. The lowest level of drug accumulation was observed in the OAW42-A resistant variant. The cellular concentration of doxorubicin after 3 hours was approximately 150 pmol/10^6 cells, representing only 25% of the level observed in the sensitive OAW42-S cells after the same time period. The effects of verapamil and cyclosporin A on doxorubicin accumulation in the variants were investigated. Verapamil reversed the accumulation defect in the three resistant cell lines and restored the cellular concentration of doxorubicin to the level observed in the sensitive

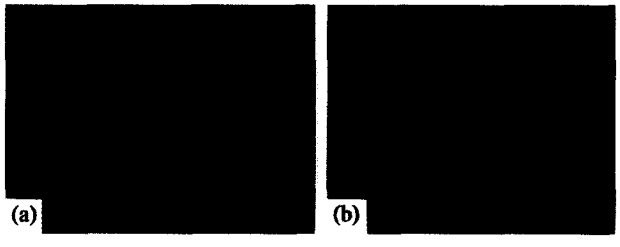

Figure 5. (a) OAW42-SR cells (passage 86) and (b) OAW42-A (passage 98) stained for vimentin with monoclonal antibody V5255 (scale bar = 25 μm).

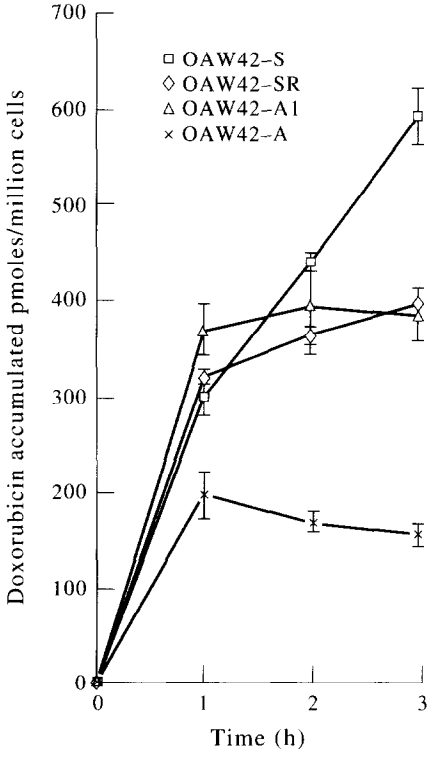

Figure 6. Doxorubicin accumulation in the OAW42 variants over a period of 3 h. The error bars represent the standard deviation from the mean results of four experiments.

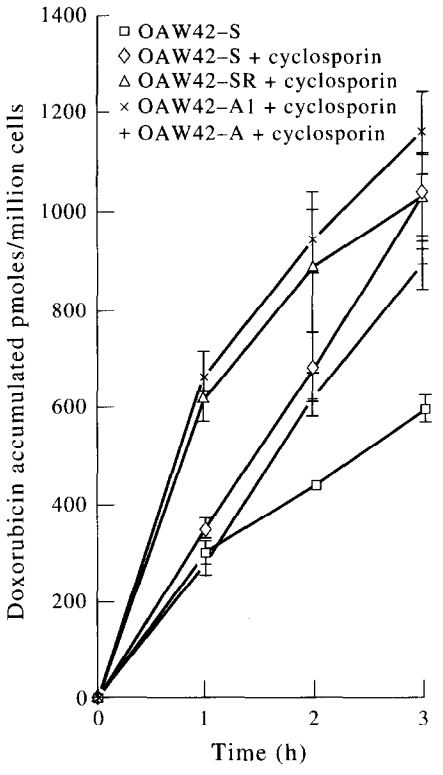

Figure 7. The effect of cyclosporin A (10 μg/ml) on doxorubicin accumulation in the OAW42 variants over a period of 3 h. The error bars represent the standard deviation from the mean results of four experiments.

OAW42-S cells (data not shown). Verapamil also caused an increase in the cellular concentration of doxorubicin in the OAW42-S cells. Cyclosporin A proved more effective than verapamil for enhancing doxorubicin accumulation in the resistant variants, causing an increase in the level of drug accumulated greater than that observed in the OAW42-S cells (Figure 7). The addition of cyclosporin A also caused an increase in doxorubicin accumulation in the OAW42-S cells.

Subcellular distribution of doxorubicin in the OAW42 variants

Figure 8 illustrates the subcellular doxorubicin distribution pattern observed in each of the four variants following 2 hours exposure to the drug. The results indicate that doxorubicin fluorescence was predominantly located in the nucleus of the OAW42-S, OAW42-SR and OAW42-A1 cells, but the intensity of nuclear fluorescence was found to be greatest in the sensitive OAW42-S cells. Cytoplasmic staining was also apparent in the OAW42-S cells. The OAW42-SR and OAW42-A1 variants displayed fluorescence of equal intensity, with regions of faint cytoplasmic fluorescence also visible. Cytoplasmic staining was less visible in the OAW42-A1 cells compared with the OAW42-SR cells. Faint nuclear fluorescence (substantially less than that seen in the OAW42-S cells) was observed in the OAW42-A cells along with faint cytoplasmic fluorescence. No significant increase in nuclear fluorescence was observed in the OAW42-A cells following longer incubation time (up to 8 hours) (data not shown). The addition of verapamil and cyclosporin A resulted in an increase in the intensity of

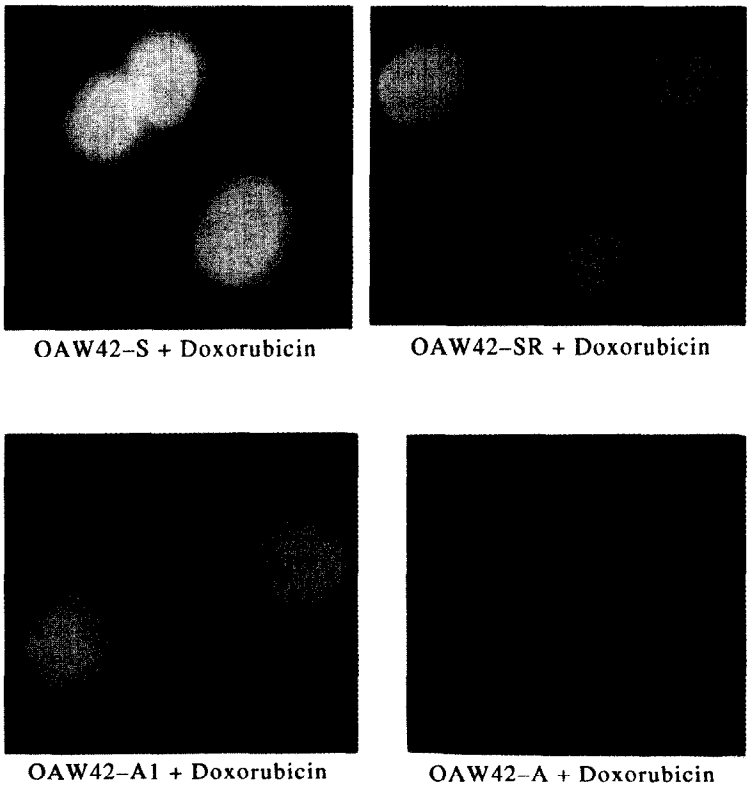

Figure 8. The subcellular distribution pattern of doxorubicin in each of the OAW42 variants following 2 h exposure to the drug (10 μM) (scale bar = 10 μm).

nuclear fluorescence in each of the resistant variants to a level greater than that observed in the OAW42-S cells, with the most significant increase in fluorescence being seen in the OAW42-A cells (data not shown).

DISCUSSION

The heterogeneity within the individual resistant variants and the differences between each of the variants was clearly demonstrated by the differential expression of the parameters investigated. The co-existence of different mechanisms of resistance in heterogeneous cell populations was highlighted particularly by the OAW42-SR and OAW42-A1 variants.

In the spontaneously resistant OAW42-SR variant, simultaneous overexpression of P-170 (at a relatively low level compared to OAW42-A1 and OAW42-A), LRP and MRP was observed. There are reports of several P-170-negative resistant cell lines which are positive for LRP, but it has also been reported that LRP and P-170 are not mutually exclusive within the same resistant tumour cell line [16], and co-expression of all three MDR-associated parameters has also been reported [17]. LRP-positive cells within the OAW42-SR cell population appeared to evolve from being a minor to a major cell population upon serial passaging of the cells. This observation suggests possible clonal selection of LRP-positive cells in long-term *in vitro* culture conditions, perhaps imitating clonal selection of resistant cell populations *in vivo*. Clonal selection of CA-125-negative ovarian cells *in vitro* has been observed by Mobus and associates [18]. The detection of increased numbers of LRP-positive cells appeared to coincide with increased resistance (doxorubicin resistance levels varied from approximately 3- to 8-fold over the sensitive clonal population OAW42-S).

The apparent change in mode of resistance from LRP- to P-170-mediated which was seen in the OAW42-A1 and OAW42-A variants may also mirror the *in vivo* development of tumour resistance from low (possibly inherent) resistance, where P-170 is undetectable, to higher levels, where P-170 is readily detectable (possibly postchemotherapy). Although the OAW42-A1 cells were only approximately 2-fold more resistant than OAW42-SR cells, a notable increase in P-170 with a corresponding decrease in LRP and MRP was observed, whilst in the OAW42-A cells which were approximately 3-fold more resistant than the OAW42-SR cells, resistance appeared to be predominantly (of the markers examined) P-170 mediated. These results indicate that LRP and MRP may be more useful markers of the lower-level resistance often encountered in the clinical setting, particularly in untreated/pretreated clinical specimens [17, 19, 20].

The investigation of LRP in very early passages of the OAW42-S cells showed that LRP was not present in these cells; likewise, P-170 was not detected. However, the positivity exhibited by later passages of these cells for LRP in the absence of any changes in toxicity profiles or drug accumulation patterns suggests the presence of a non-functional form of LRP. Studies are currently underway in this laboratory to investigate any difference between the LRP detectable on OAW42-SR cells and that detected on OAW42-S cells using monoclonal antibodies raised to both cell lines. No significant amount of P-170 was detected in these later passages of OAW42-S cells.

Only one method used for the detection of MRP (i.e. ECL-detected Western blots) indicated that this mode of resistance may make a contribution to resistance in the variants. The MRP levels detected in the OAW42-S and OAW42-SR cells may contribute to the inherent level of resistance in these cells; the increased resistance of the OAW42-SR cells relative to the OAW42-S cells may reflect the expression of P-170 in these cells. It has been suggested that the resistance of certain cells to drugs may not be a simple function of the level of MRP, but may be influenced by post-translational modifications of MRP [17, 21]. From our studies, using RT-PCR for measurement of MRP mRNA, it is apparent that levels of MRP mRNA do not always correlate with levels of MRP protein expressed [22], and highlights the need to examine all resistance-associated parameters at both the mRNA and protein levels [23]. Immunocytochemical studies were particularly unsatisfactory on cytospins of the cell lines, and work is ongoing to try to optimise conditions for MRP staining on these cells as different conditions are obviously required to those for the positive control cell line which showed definitive positive staining. Use of different detection methods and detection methods of differing sensitivities for the determination of protein levels is clearly necessary to obtain accurate data on all resistance parameters.

A decrease in levels of topoisomerase II and in particular the alpha isoform was seen in the OAW42-SR and OAW42-A1 variants indicating that four different MDR-associated parameters co-exist in the OAW42-SR cells (i.e. LRP, MRP, low-level P-170 and reduced Topo IIα) and in the OAW42-A1 cells (i.e. P-170, very low-level LRP, low-level MRP and reduced Topo IIα). The co-existence of P-170 MDR phenotype (typical MDR) and altered topoisomerase II levels (atypical MDR) within the same cell line has also been reported by Friche and associates [24]. This group of researchers found that both the α and β isoforms of the enzyme were reduced in daunorubicin-resistant Ehrlich ascites tumour cells, and suggested that, if a single cell line can show co-expression of MDR parameters, then perhaps multiple mechanisms of resistance can co-exist in tumours *in vivo*. However, in the OAW42-A variant, which showed the greatest levels of P-170 overexpression, there appeared to be no significant deficiency in topoisomerase IIα or β levels. According to Hoffmann and Mattern [25], a number of cultured cell lines overexpress P-170 but have no topoisomerase II deficiencies. No significant alterations in GSTπ levels were detected in the resistant variants compared with the sensitive OAW42-S cells. This result was not surprising as changes in GSTπ levels in doxorubicin-selected MDR cell lines have been reported to be rare by Moscow and Dixon [26]. Drug (doxorubicin) accumulation studies in the variants were predictably related to the resistance levels as determined by toxicity assays and the levels of P-170 detected in the variants. However, in some of the OAW42 variants, the reduction in doxorubicin accumulation does not appear to correlate directly with the level of P-170 detected; although drug accumulation was similar for the OAW42-SR and the OAW42-A1 variants, a lower level of P-170 was detected in the OAW42-SR cells, implying that MRP and possibly LRP may also be responsible for the drug accumulation defect mechanisms [16, 22]. Drug distribution in the OAW42 variants was shown to be inversely related to the level of doxorubicin resistance in each variant. Localisation of drug in the nucleus was seen to be greatest in the sensitive OAW42-S cells. Differential cytoplasmic flu-

orescence was also visible in resistant OAW42 cell lines, possibly representing the localisation of drug in cytoplasmic vesicles in resistant cells [27].

Cytokeratin 8 and vimentin co-expression in ovarian cell lines and tumours is well documented [18, 28, 29], but the relationship, if any, between levels of drug resistance and levels of intermediate filaments still requires clarification. Serous tumours (OAW42 cells were derived from such a tumour) with a high or moderate degree of differentiation often exhibit strong vimentin staining, and cytokeratins are the hallmark intermediate filaments of epithelial differentiation. The differential expression (increasing levels with increasing drug resistance) of the two intermediate filament proteins studied in the OAW42 variants may indicate that certain cell types (perhaps better differentiated) may be more susceptible to becoming resistant and subsequently evolve as the major cell population during normal growth or exposure to drug. A similar increase in vimentin expression (but not in cytokeratin levels) with increasing drug resistance levels was observed by Conforti and associates [30] in human colon carcinoma cell clones (LoVo).

In conclusion, the OAW42 variants described here may be useful cell models for inherent and low-level drug-induced resistance. Moreover, the results described above indicate the necessity for the investigation of several MDR-related parameters in clinical specimens in order to understand the underlying mechanisms of resistance.

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