Sequential Coexpression of the Multidrug Resistance Genes MRP and mdr1 and Their Products in VP-16 (Etoposide)-selected H69 Small Cell Lung Cancer Cells

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Abstract

Resistance to drugs included in the multidrug-resistance phenotype has been attributed to overexpression of either mdr1 or MRP genes and their products in numerous cell lines, while coexpression, to our knowledge, has not previously been reported in the same cells. Human small cell lung cancer H69/VP cells were developed by continuous incubation in increasing doses of VP-16. In reverse transcription-PCR assays we found overexpression of both mdr1 and multidrug-resistance protein (MRP) genes, and immunoblots showed both elevated P-glycoprotein and MRP in H69/VP cells. Double immunocytochemical staining demonstrated the expression of both MRP and P-glycoprotein in the same cells, indicating that the observations do not result from the selection of two independent clones. Examination of early passages of H69/VP cells showed that overexpression of MRP mRNA occurred prior to mdr1. Thus, cell lines and clinical samples in the future should be tested for both mdr1/P-glycoprotein and MRP since a positive result for one of the phenotypes does not preclude the existence of the other.

Introduction

SCLC is a disease in which VP-16 is one of the most important drugs in achieving remission rates of above 80% in first-line treatment (1). At relapse, however, tumors are usually resistant to treatment. Consequently, there is considerable interest in detecting mechanisms of resistance to VP-16 in SCLC. There are at present two well-defined MDR phenotypes which include VP-16 and which are both due to the expression of ATP-binding transmembrane proteins, namely, P-gp coded for by mdr1 and MRP coded for by the MRP gene (2, 3). Several MDR SCLC cell lines, as well as a large number of cell lines derived from other cancers, have been described which overexpress either mdr1/P-gp or MRP, but none to our knowledge which overexpress both. In this study we present a SCLC line selected in VP-16 which sequentially overexpresses both MDR genes.

Materials and Methods

Cell Lines. The human SCLC line H69 (4) was maintained in RPMI 1640 containing 10% FCS in a 7.5% humidified atmosphere. At passage 70 H69 cells were exposed to increasing concentrations of VP-16, starting at 0.5 μM for 3 passages, followed by 1.0 μM for 4 passages (to passage 77), 1.5 μM for 5 passages (to passage 82), 2.0 μM for 5 passages, 2.5 μM for 6 passages (to passage 93), and finally at 3.0 μM to passage 125. Cells were frozen at passage 77, 82, 93, 108, 122, and 125. The cross-resistance pattern of H69/VP in clonogenic assay at passage 125 has been described in detail (5, 6), and H69/VP cells are 17-fold resistant to VP-16 and 8- and 6-fold cross-resistant to vincristine and doxorubicin, respectively. H69/AR cells selected in doxorubicin and which overexpress MRP only (3) and 8226/DOX cells which overexpress mdr1/P-gp only (7) were used as positive controls.

RT-PCR. mRNA was obtained from 5 × 10⁶ cells using the oligotex mRNA kit (QIAGEN, Hilden, Germany). Yield and purity of the mRNA were checked by spectrophotometric determination at 260 and 280 nm. First-strand cDNA synthesis was performed on 0.2 ng mRNA by reverse transcription using the random primer extension method. The mRNA was incubated with 24 units avian myeloblastosis virus RT enzyme, 5 μl random hexanucleotide (62.5 units/ml), 24 units RNase inhibitor, 1 mM dNTP, and 10 μl Sx RT buffer (all reagents from Boehringer-Mannheim, Mannheim, Germany) in a final reaction volume of 50 μl. After incubation at 37°C for 1 h the reaction was terminated by heating at 94°C for 5 min, whereafter the cDNA was diluted 1:2 in sterile water and stored at ~20°C until use. All sequences for PCR primer sets have previously been described, namely, the 167-base pair mdr1-specific and 120-base pair β-microglobulin; P-gp primer sequences in (6), and the two different MRP primer pairs for the 237-base pair sequence (9) and the 596-base pair sequence (10). The mdr1 and MRP primer sets used span an intron to control against amplification of genomic DNA sequences. The PCR reactions were performed using a fixed input of cDNA and primers optimized for each set of primers as follows: for β-m, cDNA derived from 10 ng mRNA and 25 pmol of each primer was used, while for mdr1 and the 237-base pair MRP primer set cDNA derived from 20 ng mRNA with 100 pmol of each primer were used. Finally, for the 596-base pair MRP primer set, cDNA derived from 20 ng mRNA was mixed with 25 pmol of each primer. Otherwise, the PCR conditions were: 200 μM dNTP, 2.5 units Termoprime Plus DNA polymerase (Advanced Biotechnology, Surrey, United Kingdom) in PCR buffer (1.5 mM MgCl₂, 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl, pH 9.0, and 0.1% Tween; Advanced Biotechnology), adjusted to a final reaction volume at 100 μl. The reaction mixtures were overlaid with a drop of mineral oil and heated to 72°C for 10 min. Amplifications were performed for 40 cycles of 94°C for 2 min, 55°C for 1 min, and 72°C for 1 min. After the last cycle all samples were incubated at 94°C for an additional 10 min. Ten μl PCR product were electrophoresed in Tris Acetate EDTA buffer (4 mM Tris, 1 mM EDTA, 20 mM Acetate, pH 8.4) on 3.5% NuSieve agarose gels (FMC BioProducts, Vällensbaek Strand, Denmark) containing ethidium bromide. The products were then visualized on a UV transilluminator and photographed.

Membrane Preparation and Immunoblotting. Membrane-enriched fractions were prepared as previously described and resuspended in Tris-sucrose buffer (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose) containing protease inhibitors (11). Membrane protein was solubilized in Laemmli buffer (12) and subjected to SDS-PAGE, electrotransfer, and immunoblotting essentially as described (13). Blots were incubated with MRP-specific mAb QCRL-1 (ascites, diluted 1:5000) (13), or with P-gp specific mAb C219 (1 μg/ml; Centocor, Malvern, PA), followed by horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (H + L), F(ab')² fragment (diluted 1:10,000, Pierce, Edmonton.

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3 The abbreviations used are: SCLC: small cell lung cancer; β-m, β₂-microglobulin; MDR, multidrug resistance; MRP, multidrug-resistance protein; VP-16, etoposide; TBS, Tris-buffered saline; P-gp, P-glycoprotein; RT, reverse transcriptase.
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Alberta, Canada). Antibody binding was determined by enhanced chemiluminescence detection (Amersham Corp., Oakville, Ontario, Canada) and exposure on Kodak X-Omat film.

Double Immunocytochemical Staining. Cytospins of H69 and H69/VP cells were air dried and fixed in 4% formaldehyde buffered in PBS (Lillie’s fixative) for 10 min followed by permeabilization for 30 min in TBS (pH 7.4) containing 1% Triton X-100. Cytospins were then washed three times in TBS followed by blocking in TBS containing 1% BSA for 10 min. Incubation of primary antibodies was performed overnight at 4°C with a mixture of monoclonal QCRL-3 anti-MRP (13) at 2 μg/ml and rabbit polyclonal mdrl ab-1 anti-Pgp (Oncogene Science, Uniondale, NY) at 10 μg/ml. Although both QCRL-1 and QCRL-3 antibodies stained formalin-fixed H69/VP cells, the reaction in our hands was more distinct for QCRL-3 which was subsequently used. Polyclonal anti-P-gp was used instead of the monoclonal C219 in the immunoblots because the double immunolabeling technique requires primary antibodies from different animal sources. The primary mAb was detected using goat anti-mouse antibody followed by peroxidase-antiperoxidase mouse antibody complexes. Polyclonal antibodies were detected by alkaline phosphatase-conjugated swine anti-rabbit antibodies. Between each incubation step cyto-

Results

RT-PCR. As shown in Fig. 1, H69/VP cells at passage 125 expressed amplification products for both MRP and mdrl while H69 cells were negative. Amplification of β-m was done in parallel, as an internal control to ensure equal integrity and amount of the cDNA produced from the two cell lines. Experiments using the 596-base pair MRP fragment primers (10) showed the same results (data not shown). Fig. 2 illustrates sequential passages of the H69/VP cell line during its challenge to the drug. Amplification of β-m was also performed in parallel to check the integrity of the cDNA obtained from the cells (data not shown). Drug challenge started at passage 70 (see “Materials and Methods”) and there was no detectable level of either mdrl or MRP after 7 passages while MRP alone was identified at passage 82. In passage 93 the PCR reaction yielded a very faint mdrl as well as a strong MRP signal, thus representing the earliest examined passage with coexpression of both mdrl and MRP genes. Passage 125 showed clearly detectable levels of both the mdrl and the MRP PCR products.

Immunoblotting and Immunostaining. Immunoblotting on H69 cells and H69/VP cells at passage 125 with MRP and P-gp-specific antibodies showed bands of M, 190,000 and 170,000, respectively, in H69/VP only, corroborating the RT-PCR results shown in Fig. 1. However, the amount of MRP in H69/VP cells was much less than in H69/AR cells, and P-gp expression was also weak compared to the 8226/Dox40 positive control (Fig. 3).

Using a double immunocytochemical stain, we could clearly identify H69/VP cells with a positive reaction for both MRP and P-gp in several independent experiments (Fig. 4). Control stains and H69 cells were negative. The stain was very heterogeneous, with a mixture of strongly and weakly positive cells which agrees with the relatively low amount of protein expressed in this cell line compared to highly overexpressing cell lines shown in Fig. 3. These experiments also indicated co-overexpression of P-gp and MRP in single H69/VP cells. Thus, these results demonstrate that this H69/VP line is not a composite of different MRP- and mdrl/P-gp-expressing clones.

Discussion

Resistance to VP-16 is a major problem in the treatment of SCLC. The expression and role of mdrl/P-gp in clinical SCLC is somewhat controversial, presumably due to the various detection methods applied to the clinical samples, and at present there is no published data on MRP overexpression in this disease (14). However, the relatively high expression of MRP in normal lung tissue (3) might imply a role for this gene in SCLC because it appears from numerous studies on mdrl/P-gp that this MDR phenotype is often expressed in tumors derived from tissues with a high expression. Selection of MDR phenotype may also be due to some extent to the selective agent. Thus while doxorubicin selected for mdrl only in a large number of MES-SA sarcoma cells, VP-16 did not (15, 16). The H69 cell line has been used in several resistance studies and has shown mdrl overexpression after daunorubicin (17), doxorubicin (18), and VP-16 (19, 20) selection and MRP after doxorubicin selection (3). Our H69/VP cell line demonstrated an energy-dependent decrease in daunorubicin accumulation which was inhibited by verapamil, and also had a cross-resistance pattern typical for P-gp-overexpressing cells (5), but we could not detect P-gp in Western blots or in azidopine photoaffinity labeling from passage 125 although this was easily seen in H69/DAU cells selected in daunorubicin (6). The present study utilized the more sensitive methods of RT-PCR and immunoblotting with enhanced chemiluminescence and showed that mdrl/P-gp was overexpressed in the later passages of H69/VP (Figs. 1–3). Furthermore, MRP was co-overexpressed (Fig. 1) and when early passages were analyzed was shown to precede mdrl overexpression (Fig. 2). This phenomenon of sequential co-overexpression of two distinct MDR genes has not previously been reported and raises some interesting aspects on the emergence of drug-resistance genes. Using classic fluctuation analysis, mdrl MDR is believed to be due to selection of preexisting mutants with a spontaneous mutation rate of approximately 10^-6 (15), although induction of mdrl has also been reported to occur (21). MRP has not yet been analyzed as to its
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development of expression. However, if overexpression of both mdrl and MRP were to be solely due to selection of mutants, then sequential co-overexpression, as in this case, would be a more likely event than simultaneous co-overexpression (22). In our H69/VP cell line, MRP was first overexpressed at a rather moderate level compared to H69/AR (Fig. 3) when the same cells apparently started to overexpress mdrl/P-gp also at very moderate levels compared to H69/DAU (6). It is therefore intriguing that H69/VP cells at passage 93 simply did not increase their MRP levels instead of increasing levels of a different resistance protein. Promoter analysis at the various passages may provide information on the differential regulation of these two MDR genes. Furthermore, fluctuation analysis on H69 cells exposed to VP-16 will be an interesting model to determine whether selection and induction exists.

The results in the present study have the practical implication that both cell lines and clinical samples should be tested for both mdrl/

P-gp and MRP since a positive result for one of these MDR genes does not preclude the existence of the other.

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References

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