Comparison of Western blot analysis and immunocytochemical detection of P-glycoprotein in multidrug resistant cells

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SUMMARY A sensitive immunocytochemical technique was developed to detect a 170 000 dalton cell membrane glycoprotein (P-gp) in cell lines resistant to vincristine and vinblastine with varying degrees of resistance. P-gp was shown very clearly using the C219 monoclonal antibody and immunocytochemical detection with either antialkaline phosphate or peroxidase-antiperoxidase with silver gold intensification. There was good correlation between the results obtained with immunocytochemical detection of P-gp in single cells and Western blot analysis.

The technique is easily performed and can detect P-gp in relatively small numbers of cells that Western blot analysis could miss and is suitable for clinical application.

The development of multidrug resistance is associated with increased expression of a gene, or family of genes, encoding a 170 000 dalton glycoprotein, P-glycoprotein (P-gp).\textsuperscript{1,2} Several lines of evidence suggest that P-gp is the energy dependent drug efflux pump responsible for multidrug resistance in cells selected in culture. The role of P-gp in the development of clinical drug resistance needs to be investigated.\textsuperscript{1,5} Increased concentrations of P-gp have been shown in multidrug resistant cultured cell lines and human tumours by Western blot analysis of cell membrane preparations using monoclonal antibodies specifically developed for P-gp.\textsuperscript{6,8} This method relies on the homogenisation and extraction of cell membranes from whole tumour tissue and would almost certainly fail to detect a multidrug resistant population that comprised only a small percentage of the tumour cells. In contrast, the immunocytochemical detection of P-gp works at the single cell level, which potentially could achieve the spatial resolution and degree of sensitivity that is required to investigate the development and emergence of multidrug resistance in heterogeneous tumours. We therefore developed a relatively simple immunocytochemical technique, using the C-219 monoclonal antibody to detect P-gp in single cells, and compared the sensitivity of this method with Western blot analysis of P-gp.

Material and methods

Human lymphoid cell lines (CCRF-CEM) with varying degrees of resistance (0–1000 ng/ml) to vinblastine and vincristine were selected for growth in the presence of increasing concentrations of drug.\textsuperscript{6} The CEM/VLB\textsuperscript{100} and CEM/VLB\textsuperscript{1000} are vinblastine resistant sublines which grow in the presence of 100 ng/ml and 1000 ng/ml vinblastine, respectively. These lines were cloned and made available for this work by Dr V Ling, Toronto, Canada. The vincristine resistant lines (CEM-VCR) and vinblastine resistant lines (CEM-VLB 10, 20, 40, 50) were cloned and kindly made available by Dr D Hedley and Dr K Holmes, Ludwig Institute for Cancer Research, Sydney, Australia. The resistant cell lines exhibit characteristic pleiotropic drug resistance.

WESTERN BLOTTING

Cells (10^5–10^6) were disrupted using a hydraulic cell disruptor and a plasma membrane fraction prepared by differential centrifugation. Solubilised membrane proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filter by Western blotting resistance as described previously.\textsuperscript{7} Immunoblots were blocked overnight with 10% v/v bovine serum albumin (BSA) at 37°C and, after washing with cold phosphate buffered saline (PBS), were probed with C219, a monoclonal antibody against P-gp, provided...
by Dr V Ling. After washing in cold PBS the filters were further incubated with biotinylated antimouse antibody followed by streptavidin linked peroxidase and the reaction developed with nitro blue tetrazolium.

**IMMUNOCYTOCHEMICAL DETECTION OF P-GP IN SINGLE CELLS**

After cytopinning on to clean glass slides cells were briefly fixed in acetone and air dried. The slides were subsequently immersed in 0.3% hydrogen peroxide in methanol for 10 minutes, rinsed in water followed by Tris buffered saline (pH 7.6) and non-immune serum before incubation with the C219 monoclonal antibody (1/125) for 60 minutes at room temperature. The slides were then exposed to rabbit antimouse immunoglobulin (Dako) (1/100) for 15 minutes. After washing, the slides were incubated with swine antirabbit immunoglobulin 1/50 (Dako) for a further 15 minutes, washed, and incubated in peroxidase antiperoxidase 1/100 (Dako). The peroxidase reaction was developed in a solution of freshly prepared dianinobenzidine (DAB) and hydrogen peroxide and the signal was amplified using the technique of silver gold intensification that has been described previously. We also explored the use of an immunoalkaline phosphatase technique for labelling P-gp. The alkaline phosphatase-antialkaline phosphatase (APAAP) technique used has been previously described in detail. Briefly, after fixing the cells in acetone the slides were washed before incubation with the C219 monoclonal antibody (1/125) for 60 minutes. The cells were then exposed to rabbit antimouse immunoglobulins (Dako, 1/50) for 30 minutes at room temperature. This was repeated and the slides were rinsed with Tris buffer before being incubated with alkaline phosphatase substrate for 20 minutes and counterstained with haematoxylin. This results in a vivid red reaction product in positively labelled cells. Controls included omitting the primary antibody from the reaction and the use of vincristine and vinblastine sensitive and resistant cell lines.
Detection of P-glycoprotein

Results

The results of studies performed on vinblastine and vincristine resistant CCRF-CEM lymphoid cells showed a good correlation between Western blot analysis and immunocytochemical detection of P-gp in single cells, particularly at higher levels of resistance (figs 1–3). It was not possible to detect confidently the presence of P-gp at levels of resistance to vincristine or vinblastine below 40 ng/ml using either technique. The sensitivity of immunocytochemical detection of P-gp was similar using either the antialkaline phosphatase method or peroxidase-antiperoxidase with silver-gold intensification. Membrane staining was predominant, but at higher levels of resistance with increased expression of P-gp, there was also evidence of cytoplasmic staining. Although there seemed to be a stepwise increase in the intensity of membrane staining in cells at the lower range of resistance, this was not the case in the more resistant cells, and cells resistant to 1000 ng/ml vinblastine/vincristine had a similar degree of membrane staining to those resistant to 100 ng/ml.

The usefulness of the immunocytochemical tech-
Determination of detection of P-gp was tested on cytospins prepared from samples of blood, or bone marrow from patients with acute leukaemia and myeloma. The preliminary results indicate that the technique is well suited to the testing of clinical samples and permits the detection of P-gp in relatively small numbers of cells that would almost certainly be missed using Western blot analysis. (Fig 4).

Discussion

Drug resistance has become a major obstacle to the successful management of cancer and accounts for the failure of chemotherapy to cure or induce long term remissions in most patients with advanced cancer. The phenomenon of multidrug resistance following exposure of a cell population to a single agent has been extensively studied using in vitro models, and there is growing evidence to show that it also occurs during the course of clinical chemotherapy. Prospective studies are needed to determine how frequently clinical drug resistance is associated with the increased expression of P-gp in human tumours. Such studies have used either Northern blotting to detect mRNA, or Western blotting to detect P-gp in tumour samples that have been homogenised and extracted. These methods sacrifice the resolution and sensitivity that could be potentially achieved with either in situ hybridisation or immunocytochemical detection of P-gp in single cells. This is essential in the examination of heterogeneous tissues where the multidrug resistant cell population may initially comprise only a small percentage of the tumour cells, as exemplified by our preliminary results in screening peripheral blood and bone marrow obtained from patients with leukaemia and myeloma.

Our studies indicate a good correlation between the results achieved with immunocytochemistry and Western blot analysis in detecting the presence of P-gp in a homogeneous cell population with levels of resistance of > 40 ng/ml for vinblastine or vincristine. It is, however, impossible to quantitate accurately the degree of resistance according to the extent of immunostaining as cells resistant to greater than 100 ng/ml vincristine/vinblastine have a very similar appearance to cells resistant to 1000 ng/ml. The immunocytochemical technique is nevertheless simple to perform and has the advantage of detecting P-gp in relatively small numbers of cells that could be missed using Western blot analysis. Studies are underway to determine whether the immunocytochemical detection of P-gp in human tumours correlates with multidrug resistance, and to define its clinical role.

References


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