IMMUNOHISTOCHEMICAL DETECTION OF P-GLYCOPROTEIN IN HUMAN TUMOR CELLS WITH A LOW DEGREE OF DRUG RESISTANCE

Henricus J. BROXTERMAN^{1,4}, Herbert M. PINEDO¹, Catharina M. KUIPER¹, Jacobus J.M. VAN DER HOEVEN¹, Pieter DE LANGE¹, Jasper J. QUAK², Rik J. SCHEPER², Hiskias G. KEIZER³, Gerrit Jan SCHUURHUIS¹ and Jan LANKELMA¹

Departments of ¹Medical Oncology, ²Pathology and ³Anthropogenetics, Free University Hospital, Amsterdam, The Netherlands.

We report the immunohistochemical detection of the 170-180 kDa multi-drug-resistance-related P-glycoprotein in human tumor cells with a low level of resistance. A series of human squamous lung cancer cell lines with increasing levels of resistance to doxorubicin (DOX) was developed and stained for P-glycoprotein, using the JSB-1 MAb. Subline SW1573/50A with a 4- to 6-fold cross-resistance to daunorubicin (DNR) and vincristine (VCR) showed rather uniform positive staining for P-glycoprotein apparently at cytoplasmic sites. Only in cells with higher degrees of resistance (> 10-fold) could plasma-membrane-associated P-glycoprotein be made visible. DNR efflux was increased in SW1573/50A as compared to the parent line SW1573 (52 and 70% DNR were retained during 3 min efflux respectively). Verapamil partially reversed DNR and VCR resistance in SW1573/50A. Cells obtained from a metastasized renal cell carcinoma and cultured *in vitro* stained in a similar way to SW1573/50A and showed some sensitivity to verapamil modulation of VCR cytotoxicity. Our results suggest that weakly resistant cancer cells obtained from patients can be routinely detected with JSB-1 on cytopspins, and implicate that in such weakly resistant cells P-glycoprotein may be present, while plasma membrane expression is not yet readily detectable.

Major progress has been made in understanding cellular mechanisms of multi-drug resistance (Gerlach *et al.*, 1986; Pastan and Gottesman, 1987). Multi-drug-resistant cells display cross-resistance to a broad range of antineoplastic agents, such as anthracyclines, Vinca alkaloids, etoposide and actinomycin D (Biedler and Peterson, 1981). The MDR phenotype is correlated with over-expression of the *mdr1* gene which codes for the transmembrane protein, P-glycoprotein (De Bruyn *et al.*, 1986) and with increased drug efflux from resistant cells (Inaba *et al.*, 1979; Broxterman *et al.*, 1988).

Since over-expression of P-glycoprotein is a major feature of the MDR phenotype (Pastan and Gottesman, 1987; De Bruyn *et al.*, 1986) it has been suggested to be a useful marker for specific detection of MDR tumor cells obtained from cancer patients. Recently, over-expression of P-glycoprotein has been investigated using immunoblotting with an MAb directed against P-glycoprotein. In that study, 6 out of 25 sarcomas, but none of 35 other tumors of different types, showed elevated P-glycoprotein levels (Gerlach *et al.*, 1987). However, since immunocytochemistry would be a more sensitive method able to detect individual P-glycoprotein-expressing cells, we have recently developed an MAb (JSB-1) against a conserved epitope of P-glycoprotein with high affinity for acetone-fixed cells on cytocentrifuge preparations (Scheper *et al.*, 1988). We now report the ability of JSB-1 to detect cells with a low degree of resistance.

MATERIAL AND METHODS

Cell lines and drugs

A series of sublines with increasing levels of resistance to doxorubicin were developed from the squamous lung cancer cell line SW1573, originally established by Dr. A. Leibowitz (Scott and White Clinic, Temple, TX) and supplied by Dr. H. Joenje (Dept. of Anthropogenetics, Free University, Amsterdam). SW1573/50A, SW1573/80 and SW1573/500 were cul-

tured in the continuous presence of DOX (Adriablastina; Farmitalia, Milan, Italy) at concentrations of 50 nM, 80 nM and 500 nM respectively; SW1573/SH was a partially revertant cell line obtained by culturing SW1573/500 without DOX for 6 months. All cells were grown in Dulbecco's modified essential medium, supplemented with 7.5% heat-inactivated fetal calf serum (Flow, Irvine, Scotland) and subcultured (Broxterman *et al.*, 1987). Cells were free of Mycoplasma as tested with Hoechst stain 33258. Daunorubicin-HCl was obtained from Specia (Paris, France) and vincristine-sulfate and verapamil-HCl from Sigma (St. Louis, MO).

Patient tumor cells

Pericardiac fluid containing adenocarcinoma cells, designated HiB1, from an unknown primary tumor was obtained from a 44-year-old woman, presenting with heart failure due to pericarditis carcinomatosa. The patient received one cycle of cyclophosphamide and doxorubicin, but showed early tumor progression. Pleural fluid containing tumor cells (HiB2) was obtained 26 days after administration of the drugs. At *post-mortem* examination the primary tumor was found to be a renal-cell carcinoma. *In vitro* HiB1 and HiB2 cells grew rapidly in clusters. Cells from passages 2-10 were used for immunohistochemical investigations with JSB-1 and to determine drug chemosensitivity profiles.

Chemosensitivity assay

A tetrazolium dye (MTT) assay was used (Mosmann, 1983). Briefly, 1,500-2,000 cells were seeded in 96-well microtiter plates in 100 μ l growth medium. After 16 hr, 100 μ l of drug solution in medium were added. After about 3 doubling times the medium was removed, and 50 μ l MTT (Sigma) in phosphate-buffered saline (0.4 mg/ml) were added. The plates were incubated for 4 hr at 37°C and 150 μ l DMSO were added to dissolve the formazan. The optical density was read on a Flow Multiscan MC plate-reader at 540 nm. All drug concentrations were tested in 4 replicate wells and each experiment was done 2 to 4 times.

Daunorubicin efflux measurements

Cells (2.5×10^5) were seeded onto glass plates and incubated overnight in growth medium at 37°C. Then the glass plate was mounted in a perfusion chamber enabling intracellular DNR to be detected (Lankelma *et al.*, 1988). Briefly, cells were equilibrated in perfusion medium for at least 30

⁴To whom reprint requests should be sent, at the Department of Medical Oncology, Free University Hospital, De Boelelaan 1118, 1081 HV Amsterdam, The Netherlands.

Abbreviations: MDR, multi-drug resistance/resistant; MAb, monoclonal antibody; DOX, doxorubicin; DNR, daunorubicin; VCR, vincristine.

TABLE I - CHEMOSENSITIVITY OF THE CELL LINES FOR DNR AND VCR WITH AND WITHOUT VERAPAMIL

Cell line	Daunorubicin		Vincristine	
	RF ¹	DMF	RF	DMF
SW1573	1	1.0±0	1	1.8±0.3
SW1573/50A	3.6±0.3	2.4±0.4	5.5±1.2	5.7
SW1573/80	8.4±4.3	2.0±0.6	15.4±3.0	5.4±2.9
SW1573/SH	20.0±1.4	5.9±0.1	68.8±2.9	ND
SW1573/500	70±14	11.3±1.8	540±56	ND
HiB2	—	1.0±0	—	2.2±0.5

¹RF = resistance factor is defined as IC₅₀ of resistant cells divided by IC₅₀ of parent SW1573 cells. DMF = dose modifying factor by 8 µM verapamil = IC₅₀ of cell line for DNR (VCR) without verapamil divided by IC₅₀ with verapamil. IC₅₀ of SW1573 is (2.5±0.7) × 10⁻⁸ M (DNR) and (1.2±0.3) × 10⁻⁹ M (VCR). IC₅₀ of HiB2 is (3.6±1.3) × 10⁻⁸ M (DNR) and (6.7±2.9) × 10⁻⁹ M (VCR). All data are means ± SD from 2-4 experiments. ND=not done.

TABLE II - FRACTION OF DAUNORUBICIN RETAINED IN THE CELLS AFTER 3 MIN EFFLUX FROM SW CELL LINES

Cell line	Fraction
SW1573	0.701±0.003
SW1573/50A	0.521±0.009
SW1573/80	0.504±0.032
SW1573/500	0.431±0.005

Results are means ± SD from 2-3 experiments.

min. For these experiments the medium was buffered with 20 mM HEPES and contained 10% fetal calf serum. The pH was set at 7.45. The cells were loaded with a short (2-min) pulse of approximately 500 µM DNR. Cellular DNR content was measured in a dynamic way using a high-performance liquid chromatography pump equipped for micro-liquid chromatography (Gilson, type 302; 5.S head, Villiers-Le-Bel, France) by monitoring DNR absorption in the cells at 480 nm with a scanning spectrophotometer (Kontron Analytical, type Uvikon 722 LC, Basel, Switzerland).

Immunohistochemistry

Cells were cytospun, air-dried overnight and then fixed for 10 min in cold (4°C) acetone. Immunostaining was done with the Histostain-SP kit (Zymed, Burlingame, CA). JSB-1 ascites was used in a dilution of 1:125 and as a negative control antibody CT-6 was used, which is also an IgG₁ (Tan *et al.*, 1985). Parallel slides were stained with MRK-16, another MAb directed at P-glycoprotein, which was obtained from Dr. T. Tsuruo (Hamada and Tsuruo, 1986). The slides were incubated with MAbs for 1 hr. Hematoxylin was used as counterstain.

RESULTS

The chemosensitivity of the cell lines to DNR and VCR is shown in Table I. The Table shows that all the cell lines display cross-resistance to DNR and VCR and that also, in the weakly resistant sublines, verapamil is able to partially reverse the resistance. Furthermore, a slight modulation of DOX (not shown) and VCR cytotoxicity by verapamil could be seen in HiB2 cells.

To further define the cause of resistance in these cell lines we measured the efflux of DNR in a micro flow-through device, which makes it possible to measure drug residing inside the cells after the cells have been loaded with a high-dose DNR and subsequent efflux of DNR (Lankelma *et al.*, 1988). In Table II, the percentage of DNR remaining in the cells after an efflux period of 3 min is given, clearly showing that DNR retention was impaired in the resistant cell lines compared to the parent line. Thus, these resistant cell lines are variants displaying an increased drug efflux, which is consistent with an MDR phenotype.

The results of the immunohistochemical staining with JSB-1 are shown in Figure 1. A clear plasma membrane staining was

seen in SW1573/500 (Fig. 1b) and SW1573/SH (not shown). SW1573/50A and SW1573/80 showed an intense cytoplasmic, possibly Golgi-related staining (Fig. 1c,d).

SW1573 cells showed a faint staining (Fig. 1a) compared to CT-6 negative controls, suggesting also some expression of P-glycoprotein in this cell line.

Figure 1e shows negative control (CT-6) stained SW1573/50A cells. All other cell lines also failed to stain for CT-6 (not shown). We also performed immunohistochemistry on HiB1 and HiB2 renal carcinoma cells, which we obtained recently from the clinic. Positive staining of these cells was seen with JSB-1 but not with CT-6 and, as in SW1573/80, this staining appeared to be cytoplasmic mainly (Fig. 1f).

Staining with MRK-16 showed qualitatively similar results to staining with JSB-1.

DISCUSSION

We have recently developed an MAb (JSB-1) with a high affinity for P-glycoprotein in cells on cytocentrifuge preparations after acetone fixation (Scheper *et al.*, 1988). We have now shown that JSB-1 can detect P-glycoprotein in weakly resistant cells, suggesting that it may be a useful antibody for P-glycoprotein detection in clinical tumor samples. Intense staining was seen in cells from a squamous carcinoma lung cell line with a resistance level as low as 4-5 for DOX, DNR and VCR compared to the parent line. In agreement with a role of P-glycoprotein in active drug efflux, we found that in the weakly resistant cell lines also, DNR efflux was faster than in the parent cell line. In the SW1573/80 cell line, only a few cells (<1%) showed clear plasma-membrane staining as in the highly-resistant SW1573/500 cells. The majority of SW1573/80 cells and SW1573/50A cells showed an apparently cytoplasmic, possibly Golgi-related staining (Willingham *et al.*, 1987). No such differences in staining patterns of cells have been reported before since, when immunoblotting techniques are used, they are not detectable (Gerlach *et al.*, 1987). However, our results suggest that this cytoplasmic staining type may correlate with the development of MDR.

An initial screening of some patient samples which we have recently obtained from the clinic showed that the same type of staining as in SW1573/80 can be seen in tumor cells, which were not treated *in vitro* with cytotoxic agents. A clear example was provided by the metastasized renal cancer cells. The finding that P-glycoprotein expression was readily detectable in renal carcinoma cells is very interesting in the light of the recent results of Thiebaut *et al.* (1987), who showed relatively high levels of P-glycoprotein expression in several normal tissues, such as the kidney, involved in excretion of metabolites and drugs.

In conclusion, we have shown that JSB-1 can readily detect P-glycoprotein on individual tumor cells of epithelial origin and that its expression goes along with increasing resistance to several anti-tumor agents. JSB-1 has also been shown to be of

A

B

C

D

E

F

FIGURE 1 - Immunohistochemical staining with JSB-1 of SW1573 (a), SW1573/500 (b), SW1573/50A (c), SW1573/80 (d) and HiB2 (f). All cells failed to stain when CT-6 was used. As an example, SW1573/50A cells are shown (e). Bar, 30 μ m.

value in detection of low-resistant human myeloma cells (Dalton *et al.*, in press). Therefore, JSB-1 seems to be a general probe for P-glycoprotein in diverse cancer types.

ACKNOWLEDGEMENT

This work was supported by the Netherlands Cancer Foundation (KWF grant 85-05).

REFERENCES

- BIEDLER, J., and PETERSON, R.H.F., Altered plasma membrane glycoconjugates of Chinese hamster cells with acquired resistance to actinomycin D, daunorubicin and vincristine. In: A.C. Sartorelli, J.S. Lazo and J.R. Bertino (eds.), *Molecular actions and targets for cancer chemotherapeutic agents*, Vol. 2, pp. 453-482, Academic Press, New York (1981).
- BROXTERMAN, H.J., KUIPER, C.M., SCHUURHUIS, G.J., VAN DER HOEVEN, J.J.M., PINEDO, H.M., and LANKELMA, J., Daunomycin accumulation in resistant tumor cells as a screening model for resistance modifying drugs: role of protein binding. *Cancer Lett.*, **35**, 87-95 (1987).
- BROXTERMAN, H.J., PINEDO, H.M., KUIPER, C.M., KAPTEIN, L.C.M., SCHUURHUIS, G.J., and LANKELMA, J., Induction by verapamil of a rapid increase in ATP consumption in multidrug-resistant tumor cells. *FASEB J.*, **2**, 2278-2282 (1988).
- DALTON, W.S., GROGAN, T.M., MELTZER, P.S., SCHEPER, R.J., DURIE, B.G.M., TAYLOR, C.W., MILLER, T.P., and SALMON, S.E., Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J. clin. Oncol.* (in press).
- DE BRUYN, M.H.L., VAN DER BLIEK, A.M., BIEDLER, J.L., and BORST, P., Amplification and disproportionate expression of five genes in three multidrug-resistant Chinese hamster lung cell lines. *Mol. cell. Biol.*, **6**, 4717-4722 (1986).
- GERLACH, J.H., BELL, D.R., KARAKOUSIS, C., SLOCUM, H.K., KARTNER, N., RUSTUM, Y.M., LING, V., and BAKER, R.M., P-glycoprotein in human sarcoma: evidence for multidrug resistance. *J. clin. Oncol.*, **5**, 1452-1460 (1987).
- GERLACH, J.H., KARTNER, N., BELL, D.R., and LING, V., Multidrug resistance. *Cancer Surv.*, **5**, 25-46 (1986).
- HAMADA, H., and TSURUO, T., Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc. nat. Acad. Sci. (Wash.)*, **83**, 7785-7789 (1986).
- INABA, M., KOBAYASHI, H., SAKURAI, Y., and JOHNSON, R.K., Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. *Cancer Res.*, **39**, 2200-2203 (1979).
- LANKELMA, J., KOOPMAN, G., VAN GRONDELLE, R., and PINEDO, H.M., In-beam light absorption measurement of anthracyclines in cells with a flow-through system. *Biochem. biophys. Acta*, **964**, 200-206 (1988).
- MOSMANN, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. immunol. Meth.*, **65**, 55-63 (1983).
- PASTAN, I.H., and GOTTESMAN, M., Multiple drug resistance in human cancer. *N. Engl. J. Med.*, **316**, 1388-1393 (1987).
- SCHEPER, R.J., BULTE, J.W.M., BRAKKEE, J.G.P., QUAK, J.J., VAN DER SCHOOT, E., BALM, A.J.M., MEIJER, C.J.L.M., BROXTERMAN, H.J., KUIPER, C.M., LANKELMA, J., and PINEDO, H.M., Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multidrug-resistance. *Int. J. Cancer*, **42**, 389-394 (1988).
- TAN, B.T.G., EKELAAR, F., LUIRINK, J., RIMMELZWAAN, G., DE JONGE, A.J.R., and SCHEPER, R.J., Production of monoclonal antibodies defining guinea pig T-cell surface markers and a strain 13 Ia-like antigen: the value of immunohistological screening. *Hybridoma*, **4**, 115-123 (1985).
- THIEBAUT, F., TSURUO, T., HAMADA, H., GOTTESMAN, M.M., PASTAN, I., and WILLINGHAM, M.C., Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. nat. Acad. Sci. (Wash.)*, **84**, 7735-7738 (1987).
- WILLINGHAM, M.C., RICHERT, N.D., CORNWELL, M.M., TSURUO, T., HAMADA, H., GOTTESMAN, M.M., and PASTAN, I., Immunocytochemical localization of P170 at the plasma membrane of multidrug-resistant human cells. *J. Histochem. Cytochem.*, **35**, 1451-1456 (1987).