Overexpression of a $M_r$ 110,000 Vesicular Protein in Non-P-Glycoprotein-mediated Multidrug Resistance¹

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Abstract

A $M_r$, 110,000 protein (p110) is overexpressed in P-glycoprotein-negative multidrug-resistant tumor cell lines of different histogenetic origins. These cell lines show an ATP-dependent drug accumulation defect, suggesting the presence of drug transporter molecules different from P-glycoprotein. Immunohistochemical staining with a p110-specific monoclonal antibody (LRP-56) showed that, like P-glycoprotein, the molecule has a high expression in normal epithelial cells and tissues chronically exposed to xenobiotics and potentially toxic agents, such as bronchial cells, cells lining the intestines, and kidney tubules. Staining of LRP-56 is primarily cytoplasmic, in a coarsely granular fashion, indicating that it reacts with a molecule closely associated with vesicular/lysoosomal structures. Involvement of p110 in the energy-dependent drug transport process present in the cell lines is unknown.

Introduction

Following exposure to chemotherapeutic drugs, tumor cells can acquire resistance to structurally and functionally unrelated compounds, termed MDR.¹ In tumor cell lines the MDR phenotype was found to be frequently associated with the overexpression of the MDRI gene, coding for Pgp. Pgp is inserted in the plasma membrane and acts as a drug efflux pump, lowering the intracellular drug concentration by energy-dependent extrusion of drugs from the cell (1). Overexpression of MDRI mRNA and Pgp has been observed in several human cancers derived from tissues that normally express Pgp, such as adrenal gland and colon, but also in tumors originating from Pgp-negative cells, such as myelomas and sarcomas (2–4). The clinical role of this type of MDR is still uncertain, and MDRI mRNA or Pgp overexpression may be clinically relevant only in certain tumor types. Recently we selected the Pgp-negative MDR cell line 2R120 by stepwise doxorubicin exposure of the SW-1573 non-small cell lung carcinoma cell line to 120 nm doxorubicin. The SW-1573/2R120 MDR cell line is characterized by energy-dependent reduction of drug accumulation and exhibits cross-resistance to vincristine, gramicidin D, and etoposide. No MDRI gene overexpression or Pgp is detectable in this cell line. In contrast, the SW-1573/2R160 subline, obtained by exposure of 2R50 cells to a slightly higher doxorubicin concentration (160 nm), displays strong MDRI gene overexpression (5–7). The objective of the present study was to search for putative transporter molecules that might account for the accumulation defect in the non-Pgp SW-1573/2R120 MDR cell line. The overexpression of mRNA coding for a putative transporter molecule in a non-Pgp MDR cell line has very recently been reported (8). Here we approached this issue by attempting to raise monoclonal antibodies identifying molecules overexpressed in the SW-1573/2R120 MDR cell line.

Materials and Methods

Cell Lines. The Pgp-negative MDR SW-1573/2R120 cell line was derived by stepwise selection with doxorubicin up to 120 nm (5). The RFs for this cell line are 4/17/45 (for doxorubicin, vincristine, and etoposide, respectively). The non-Pgp revertant 2R120 Rev was then obtained by omitting doxorubicin from the culture medium for over 9 months (RFs 3/2/8), whereas the Pgp-positive MDR SW-1573/2R160 cell line was obtained from 2R50 by selection with a doxorubicin concentration of 160 nm (RFs 35/480/1203). Occasional use was made of the 1R500 Pgp-positive cell line, which had been selected from SW-1573 at a 500 nm doxorubicin concentration (9), and displays a slightly higher level of MDR than the 2R160 cell line used in most experiments. The non-Pgp MDR SW-1573-derived fusant cell lines F6.1, F6.2, F6.3, and F6.4 were obtained by somatic cell fusions between lethally γ-irradiated, resistant donor SW-1573 cells and drug-sensitive SW-1573 acceptor cells. These fusants showed drug accumulation defects (10).

The SW-1573/1.1. MDR transfectant shows strong MDRI mRNA expression and intermediate immunocytochemical staining for Pgp. The transfected cell line SW-1573 S1 was used in the expression construct pCMVMDRI containing a full-length wild type MDR1 complementary DNA under control of the CMV promoter (11).

Pgp-negative MDR cell lines were derived by stepwise selection with doxorubicin [RFs for doxorubicin, vincristine, and etoposide, respectively: GLC4/ADR lung cancer cell line, 122/1866 (12); HT1080/DR4 fibrosarcoma cell line, 222/25/837 (13)] or with mitoxantrone [MCFT/Mitox breast carcinoma cell line, 8/22/16 (14); 8226/MR40 myeloma cell line; RFs for doxorubicin and mitoxantrone, respectively, 4/12 (15)].

Pgp-positive MDR cell lines were derived by stepwise selection with doxorubicin. RFs: A2780/ADR ovary carcinoma cell line, 75/190/11 (14); A2780/ADR ovary carcinoma cell line, 160 (dorozubicin (16)); H134AD ovary carcinoma cell line, RF 250 [dorozubicin (17)]; 8226/Dox40 myeloma cell line, RF 326 [dorozubicin (18)].

Monoclonal Antibody Production. BALB/c mice received injections of 1.5 × 10⁶ 2R120 cells in complete Freund’s adjuvant (Difco, Detroit, MI) into footpads of hind legs and after 18 days booster injections with 1.0 × 10⁶ 2R120 cells in PBS into both footpads. Three days later draining popliteal lymph nodes were isolated. Lymph node cells and SP2/0 myeloma cells were fused as described earlier (19). Hybrid cells secreting antibodies of interest were selected by enzyme-linked immunosorbent assay with acetone-fixed (5 min, 70%) 2R120 (strong/positive staining) and SW-1573 (weak/negative staining)-coated 96-well plates. The LR-56 hybridoma was subcloned three times by limiting dilution. LR-56 immunoglobulin was purified from ascites by protein A-Sepharose affinity chromatography.

Immunohistochemistry. Cytocentrifuge preparations of tumor cell lines were air-dried, fixed in acetone for 5 min, and incubated for 60 min with culture supernatants. Subsequently staining was visualized using an avidin-

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4The abbreviations used are: MDR, multidrug resistance; Pgp, P-glycoprotein; RF, resistance factor (concentration of drug inhibiting growth of resistant cells to 50% divided by concentration of drug inhibiting growth of the parent cells to 50%); PBS, phosphate-buffered saline.

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bacterial complex immunoperoxidase method as described earlier (2). Percentages of cells stained with the antibody LRP-56 or with the Pgp-specific monoclonal antibodies JSB-1 or C219 (19) were determined by counting at least 200 cells/preparation, whereas positive cells were classified as either weakly, intermediately, or strongly staining.

To study reactivity of normal human tissues with the LRP-56 monoclonal antibody, cryostat sections of the tissue samples were cut 5 μm thick, fixed in acetone at room temperature, and air-dried directly prior to staining. Sections 5 μm thick were also obtained from tissue blocks fixed in 10% neutral formalin and embedded in paraffin. The slides were incubated with LRP-56 (1:100 dilution, 60 min) and control antibodies, followed by incubation with rabbit anti-mouse immunoperoxidase (Dako, Copenhagen, Denmark; 1:25, 30 min), and developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO): 0.5 mg/ml PBS containing 0.015% H2O2.

**Immunoprecipitation Studies.** Tumor cells were preincubated for 2 h with minimal essential medium without methionine (Gibco, Ltd., Paisley, Scotland) and without fetal calf serum before labeling. Cells were then labeled for 16 h with 4 μCi/ml [35S]methionine (specific activity, >1000 μCi/mmol; Amersham UK) in minimal essential medium without methionine with 10% fetal calf serum. The cells were homogenized in lysis buffer (PBS with 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma)) and 1% Nonidet P-40. Nuclei and other large debris were removed by centrifugation for 5 min at 9000 × g. Supernatant aliquots containing approximately 15 × 10⁶ cpm were diluted with RPMI (Gibco) containing 1% Nutridoma supplement (Boehringer Mannheim, Mannheim, Germany) to reduce nonspecific binding and incubated for 2 h at 4°C with 15 μg LRP-56 antibody (total volume, 1 ml). For Pgp precipitation, sodium dodecyl sulfate was added to a final concentration of 0.1%. Immune complexes were then precipitated by further incubation (1 h, 4°C) with 100 μl protein A-Sepharose (CL-4B, diluted 1:1 in PBS; Pharmacia, Uppsala, Sweden). Precipitates were washed twice with lysis buffer containing 2% bovine serum albumin and 4 times with PBS, taken up in 50 μl sample buffer [200 mM Tris-HCl (pH 6.8), 1% 2-mercaptoethanol, 8% sodium dodecyl sulfate, 10% glycerol, and 0.05% bromophenol blue], and microfuged. Supernatants were loaded for overnight polyacrylamide (4–12%) gel electrophoresis (8 mA), after which the gels were stained with Coomassie Brilliant Blue (Pharmacia), and incubated (20 min, 20°C) with NAMP100 amplifier (Amer- sham, Little Chalfont, United Kingdom). Dried gels were exposed to Kodak X-AR5 (preflashed) film at −80°C for 72 h.

To study possible glycosylation of p110 additional precipitations were performed with lysates of SW-1573/2R120 and HT1080/DR4 cells, incubated with [35S]methionine in the presence of tunicamycin (0.018 μmol; Sigma; preventing N-glycosylation) or phenyl-N-acetyl-o-D-galactosamine (2.5 mM; Sigma; preventing O-glycosylation). Presence of sizable sugar moieties was also studied by postprecipitation treatments with N-glycosidase F (0.1 unit/100 μl; Boehringer Mannheim), or a mixture of neuraminidase (3 milliunits/100 μl) and O-glycosidase (1 milliunit/100 μl; Boehringer Mannheim).

**Results and Discussion**

Using the 2R120 cell line for monoclonal antibody production, we selected an IgG2b monoclonal antibody (LRP-56) for strong immunocytochemical reactivity with 2R120 (Fig. 1b) compared to the parental SW-1573 cell line (Fig. 1a), whereas only 1–3% of the cells from the Pgp-positive 2R160 cell line stained positive (Fig. 1c). Staining of LRP-56 was primarily in the outer cytoplasmic zone, in a granular fashion (Fig. 1d), suggesting that it reacts with molecule(s) closely associated with endoplasmic reticular/lysosomal structures. Staining of the plasma membrane was not prominent. Several other SW-1573-derived cell lines were studied to establish a possible relationship between non-Pgp-mediated MDR and LRP-56 staining: (a) 2R120 cells cultured without drug for 2–4 weeks still exhibited strong immunocytochemical reactivity, showing that staining was not related to acute exposure to drugs; (b) the revertant cell line 2R120-Rev, which exhibited only a low degree of residual drug resistance after culturing without drug for over 9 months, had almost returned to parental staining level (Table 1); (c) hybrid SW-1573 derived cell lines obtained by fusion of parental cells with non-Pgp MDR cells stained positive with LRP-56. After transfer of genomic DNA these fusant cell lines obtained the MDR phenotype and a drug accumulation defect, without expressing MDR1 Pgp mRNA (10). In contrast, the Pgp transfectant cell line SW-1573 1.1 which overexpresses the *MDR1* gene did not show increased LRP-56 staining (Table 1). Up-regulation of the molecule(s) detected by LRP-56 was found to be

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**Fig. 1.** LRP-56 staining of cytospin preparations of parental SW-1573 cells (a), 2R120 non-Pgp MDR cells (b, d), 2R160 Pgp-positive cells (c), and MCF7/D40 Pgp-positive MDR breast cancer cells (e). The MCF7/D40 MDR tumor cells were counterstained with C219 to show plasma membrane-bound Pgp (blue staining). Bar, 50 μm.
Table 1 Detection of Pgp and p110 in various SW-1573 (human lung cancer) cell lines by JSB-1 and LRP-56 monoclonal antibodies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pgp</th>
<th>p110</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-1573</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2R120</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2R160</td>
<td>+++</td>
<td>-/+</td>
</tr>
<tr>
<td>2R120 Rev (non-Pgp revertant)</td>
<td>-</td>
<td>+/+</td>
</tr>
<tr>
<td>F6.1-4 (non-Pgp fusing)</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>SW-1573 1.1 (Pgp transfecant)</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Cytochrome preparations were scored according to the following scale: no. –; weak. +; intermediate. ++; strong. +++.

Table 2 LRP-56 staining of various couples of parental, sensitive tumor cell lines and MDR tumor cell lines with drug accumulation defects

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Pgp-negative</th>
<th>Parental line</th>
<th>MDR line</th>
<th>Parental MDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>SW-1573</td>
<td>2R120</td>
<td>- (10)*</td>
<td>+++ (86)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>MCF7</td>
<td>HT1080/DR4</td>
<td>+ (96)</td>
<td>+++ (96)</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>HT1080</td>
<td>GLC4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Myeloma</td>
<td>8226/ADR</td>
<td>8226/MR40</td>
<td>-</td>
<td>-/+</td>
</tr>
</tbody>
</table>

Staining was scored according to the following scale: no. –; weak. +; intermediate. ++; strong. +++.

a frequent event, since three SW-1573 cell lines, independently selected for MDR by culturing in low levels of doxorubicin, all had increased staining compared to parental cells (data not shown).

Besides SW-1573 lung cancer-derived cell lines, several other non-Pgp MDR cell lines showing drug accumulation defects have been recently described, originating from different histogenetic origins. These include the GLC4/ADR small cell, lung cancer cell line (12), and cell lines derived from breast cancer [MCF7/Mitox (14)], fibrosarcoma [HT1080/DR4 (13)], and myeloma [8226/MR40 (15)]. These lines do not overexpress MDR1 and therefore might be candidates for expressing the protein(s) recognized by LRP-56. Staining of these cell lines with LRP-56 showed cytoplasmic, granular staining patterns similar to the 2R120 cells. Again, staining of the respective parental cell lines was negative or weak (Table 2). In certain instances, drug-resistant cell lines known to express Pgp (MCF7/D40) were also observed to stain positively with LRP-56 (Table 2; Fig. 1e). Apparently, Pgp-mediated MDR and LRP-56 staining are not mutually exclusive within the same resistant tumor cell line.

Immunoprecipitation studies with LRP-56 and four different non-Pgp MDR cell lines (SW-1573/2R120, HT1080/DR4, MCF7/Mitox, and GLC4/ADR) demonstrated a M, 110,000 molecule in all four cell lines (Fig. 2). Although the M, 110,000 band was expressed in certain parental cell lines, in all non-Pgp MDR cell lines it was overexpressed. Precipitations with lysates of SW-1573/2R120 and HT1080/DR4 cells, incubated with $^{[35]}$S]methionine in the presence of tunicamycin or phenyl-N-acetyl-D-glucosaminide did not lead to a detectable shift in molecular weight of the M, 110,000 band, showing that large N-linked or O-linked carbohydrates are not attached to this molecule. Absence of sizable sugar moieties was confirmed by post-precipitation treatments with W-glycosidase F or a mixture of neuraminidase and O-glycosidase, which also did not lead to reduction in molecular weight.

Because expression in normal tissues might provide information regarding the physiological role of the protein recognized by LRP-56, immunohistochemical studies of certain human tissues were performed. Strong staining was observed in aceto-ined-fixed, frozen sections and, to a lower degree, also in formalin-fixed paraffin tissue sections. Interestingly, LRP-56 staining revealed staining patterns similar to those we and others have described earlier for Pgp expression (2, 20, 21). Thus, LRP-56 staining was most pronounced in epithelial cell/tissues chronically exposed to xenobiotic, potentially toxic agents, such as bronchial cells (Fig. 3c), cells lining the intestines. 

Fig. 2. Immunoprecipitation with LRP-56 of the parental (SW-1573), non-Pgp MDR (2R120), and Pgp-positive (IR500) MDR cell lines (Lanes 1, 2, and 3). Results for the HT1080, MCF-7, and GLC-4 cells are shown in Lanes 4, 6, and 8 (parental cell lines) and 5, 7, and 9 (non-Pgp MDR counterparts). Lanes 10 and 11, control runs with C219 precipitates from the SW-1573 and Pgp-positive IR500 cell lines, respectively. No bands were overexpressed when C219 or JSB-1 were used for immunoprecipitations in any of the non-Pgp MDR cell lines. kD, molecular weight in thousands.
tines (Fig. 3b), and most tubules of the kidney (Fig. 3a). Moreover, as for Pgp, strong staining was observed in adrenocortical cells (Fig. 3d). Differences in the distribution of Pgp and LRP-56 staining were also noted, however. Whereas Pgp staining is chiefly at the plasma membrane, LRP-56 staining was primarily intracytoplasmic and coarsely granular. Differences were also noted for distinct tissue types. For example, in contrast to the Pgp-reactive antibodies JSB-1 and C219, LRP-56 did not stain liver bile canaliculi. On the other hand, LRP-56 strongly stained stratified epithelia, such as squamous and transitional epithelium. Collectively, these staining patterns suggest that the protein recognized by LRP-56, like Pgp, may be involved in a molecular transport mechanism. Its molecular weight of 110,000 argues against the possibility that LRP-56 might recognize the newly described multidrug resistance-associated protein, overexpressed in certain non-Pgp MDR cell lines, showing a single open reading frame of 1522 amino acids (8).

In conclusion, using a newly developed monoclonal antibody, we have demonstrated the overexpression of a Mε, 110,000 protein, shared by different non-Pgp MDR tumor cell lines showing drug accumulation defects. Transfer of the non-Pgp MDR phenotype to sensitive recipient SW-1573 lung cancer cells by cell fusion also resulted in LRP-56 staining, whereas staining was reduced in a drug-sensitive, partially reverted cell line. P110 is strongly expressed in nonmalignant epithelial cells with secretory or excretory functions. Staining of LRP-56 is primarily cytoplasmic, in a coarsely granular fashion, indicating that it reacts with a molecule closely associated with vesicular/lysosomal structures. It is tempting to speculate that, like Pgp, the molecule recognized by LRP-56 is related to an outward drug transport mechanism. Further gene cloning studies are required to verify this hypothesis.

References

Fig. 3. Reactivity of paraffin sections of normal human tissues with the LRP-56 monoclonal antibody. (a) kidney; (b) colon; (c) bronchus; (d) adrenal.


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