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SHORT COMMUNICATIONS

Drug resistance dependent on different molecular size P-glycoproteins in Yoshida rat ascites hepatoma cells

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Multidrug resistance is defined by resistance to structurally unrelated antitumor drugs and decreased accumulation and enhanced efflux of drugs resulting from P-glycoproteins encoded by the *mdr* genes [1–4]. Many investigators have established multidrug-resistant cell lines and studied them [5–11], but there is little evidence for naturally acquired multidrug resistance.

There are many rat ascites hepatoma (AH*) cell lines that were induced by dimethylaminoazobenzene and established as transplantable tumors [12]. Inaba *et al.* [13] and Wakusawa *et al.* [14] have reported the low sensitivity of AH66 cells to VBL resulting from a high efflux of the drug in an energy-dependent manner. This study indicates that the AH cell lines have multidrug resistance dependent upon the P-glycoprotein overexpressed in the plasma membrane.

Materials and Methods

Chemicals. VBL (Shionogi & Co., Osaka, Japan), [^{3}H]-VBL (374 GBq/mmol) and [^{3}H]azidopine (1.92 TBq/mmol, Amersham International, Amersham, U.K.) were purchased from commercial sources.

Animals and tumor cells. The AH44, AH66, AH66F and AH109A cell lines were supplied by the Sasaki Institute, Tokyo, Japan. Cells were passaged weekly through female Donryu rats weighing 100–150 g (Nippon SCL, Hamamatsu, Japan) and harvested from the tumor-bearing rats 6–10 days after transplantation.

Cell culture. Cells were suspended in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum and cultured at 37° in a CO_2 incubator.

Photoaffinity labeling. Plasma membranes, prepared by a Percoll sedimentation method as reported previously [15], were incubated with 200 nM [^{3}H]azidopine for 30 min at room temperature in the presence or absence of VBL and irradiated at 366 nm for 20 min. The sample was solubilized in SDS and 8 M urea and fractionated by electrophoresis on a 7.5% polyacrylamide/4.5 M urea gel, with a stacking gel. After being fixed and dried, the gel was autoradiographed on a Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY, U.S.A.) with an intensifying screen at −70°.

Immunostaining. Fractionated membrane proteins on the gel were transferred onto a nitrocellulose membrane filter (Schleicher & Schuell, Dassel, Germany). The filter was blocked with 3% gelatin in phosphate-buffered saline (pH 7.4) for 2 hr at room temperature and then incubated overnight with 1 μg/mL of monoclonal antibody against P-glycoprotein (C219, Centocor, Inc., Malvern, PA, U.S.A.) at 4°, which was originally isolated by Kartner *et al.* [16]. The filter paper was washed and then incubated for 1 hr with horseradish peroxidase-conjugated anti-mouse IgG (Organon Teknika Corp., West Chester, PA, U.S.A.). After extensive washing with phosphate-buffered saline

* Abbreviations: AH, ascites hepatoma; VBL, vinblastine; SDS, sodium dodecyl sulfate.

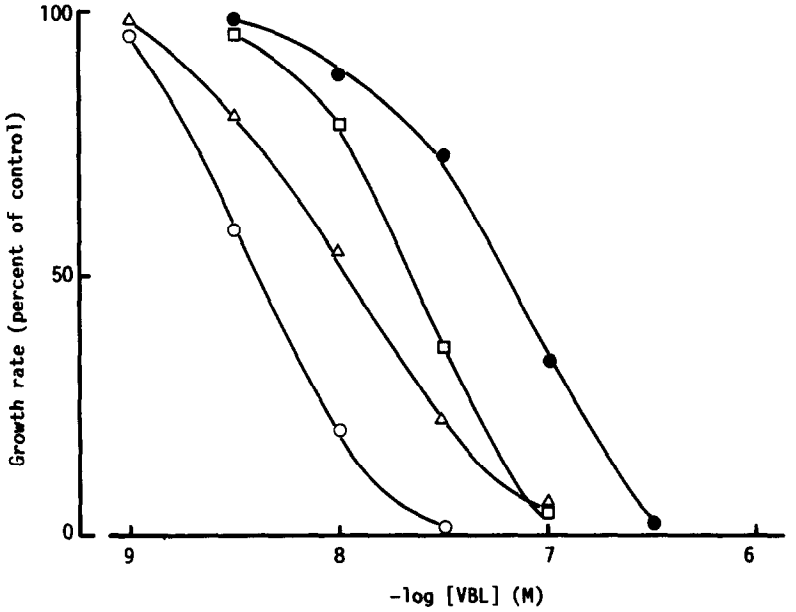

Fig. 1. *In vitro* sensitivities of AH cells to VBL. Cells (1.5×10^5/mL) were treated with VBL for 48 hr. Each point represents the mean of triplicate measurements. (△) AH44; (●) AH66; (○) AH66F; (□) AH109A.

containing 0.05% Tween 20, the immunoreactive band was stained by 0.5 μg/mL of 3,3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) and 0.03% hydrogen peroxide solution.

Results and Discussion

The *in vitro* sensitivity to VBL had the order of AH66F > AH44 > AH109A > AH66 cells. The resistance of AH44, AH109A and AH66 cells was 2.9-, 6.2- and 18.5-fold higher than AH66F cells, respectively, and AH66 cells were inherently resistant to VBL (Fig. 1). Figure 2 shows the courses of [^{3}H]VBL accumulation and efflux in AH cells. Resistant cells accumulated much less VBL than sensitive cells. The amount of VBL accumulated in AH cells in 30 min under energy-deprived conditions increased by about 2-fold (in the most sensitive AH66F cells) to 10-fold (in the most resistant AH66 cells) of that under normal conditions (data not shown). After the forced accumulation, the cells were returned to normal conditions, then the antitumor drug was rapidly extruded, according to the drug resistance. This indicates that the drug resistance in AH cells is based on the lowered accumulation resulting from the energy-dependent efflux of the antitumor drug from the cells.

It has been reported that azidopine, a photoreactive calcium channel blocker, binds irreversibly to P-glycoprotein in plasma membranes of multidrug-resistant cells [17]. The 150 and 160 kDa membrane proteins from AH109A and AH66 cells, respectively, were photolabeled by [^{3}H]azidopine. The photolabel was selectively inhibited by 10 μM VBL, more strongly in AH66 cells than in AH109A cells (Fig. 3A). The difference of affinity for VBL

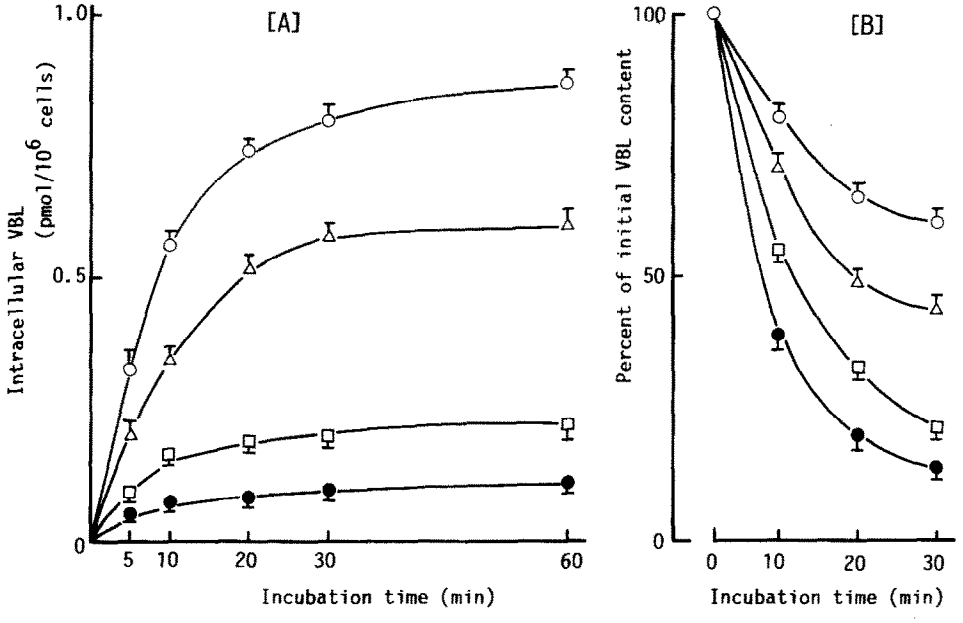

Fig. 2. Accumulation and efflux of VBL in AH cells. (A) Accumulation of VBL. Cells (1 × 10^6/mL) were incubated with 10 nM [^{3}H]VBL for the designated times at 37°. (B) Efflux of VBL. Cells (2 × 10^6/mL) were preloaded with 20 nM [^{3}H]VBL in a glucose-deprived Hanks' solution containing 10 mM sodium azide for 30 min at 30° and then cultured in the normal culture medium for the indicated times. The amount of intracellular drug was determined as described previously [11]. Bars, SE for at least two experiments performed in triplicate. Symbols as Fig. 1.

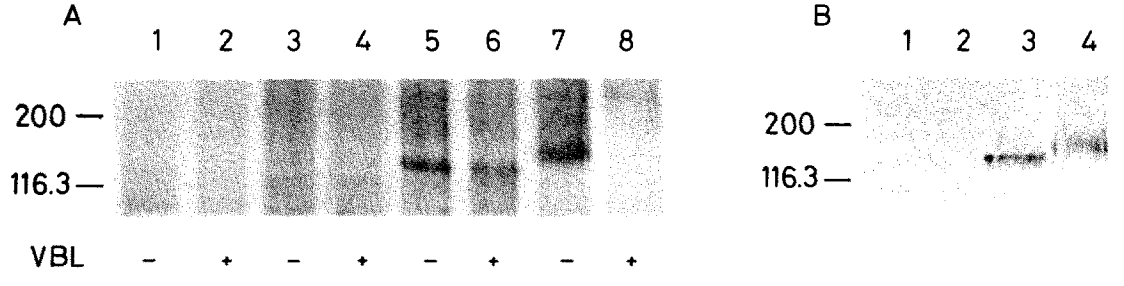

Fig. 3. (A) Autoradiogram of photoaffinity labeling of AH cell membranes by [^{3}H]azidopine. Membrane preparations (100 μg of protein per lane) from AH66F (lanes 1 and 2), AH44 (lanes 3 and 4), AH109A (lanes 5 and 6), and AH66 (lanes 7 and 8) were incubated with 200 nM [^{3}H]azidopine in the absence (−) or presence (+) of 10 μM VBL and applied on SDS-PAGE. (B) Immunostaining of AH cell membrane with C219 antibody. Membrane preparations (100 μg of protein per lane) from AH66F (lane 1), AH44 (lane 2), AH109A (lane 3) and AH66 (lane 4) were subjected to SDS-PAGE and immunoblotting with C219 monoclonal antibody. Molecular size markers are indicated in kilodaltons.

of the proteins seems to cause the difference in the magnitude of resistance between these cell lines. These proteins were also immunopositive to an antibody against P-glycoprotein C219 (Fig. 3B). In the case of the sensitive cell lines AH66F and AH44, such membrane protein bands were not detectable by photolabeling and immunoblotting. Thus, the drug resistance in AH109A and AH66 cells is dependent on 150 and 160 kDA P-glycoproteins overexpressed in the plasma membranes, respectively. The difference in molecular size of the P-glycoprotein from AH109A and AH66 cells may be due to a difference in individual glycosyl chains.

Recently, it has been reported that 170 kDa P-glycoprotein and the *mdr* gene are greatly overexpressed during treatment with hepatocarcinogens and liver regeneration [18, 19]. The AH cell lines had been induced by dimethylaminoazobenzene and passaged *in vivo* for a long period [12], so the P-glycoproteins overexpressed in AH109A and AH66 cell membranes are clearly different from an acute expression. If hepatoma cells constantly overexpress the P-glycoprotein, all AH cells have to express the protein in the membrane, but AH44 and AH66F cells had a very low level or none. Therefore, AH66 and AH109A cells can be considered to have naturally acquired multidrug resistance.

This study indicates that Yoshida rat ascites hepatoma cell lines have different sensitivities to VBL, and AH109A and AH66 cells are naturally acquired multidrug-resistant cell lines dependent upon P-glycoproteins with different molecular size.

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