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Detection of Low-level Expression of P-Glycoprotein in ACHN Renal Adenocarcinoma Cells

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A highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay and a flow cytometric assay were used to examine ACHN cells for the expression of P-glycoprotein. The expression of P-glycoprotein was detected at the RNA and protein levels in ACHN cells by RT-PCR and flow cytometry, respectively. However, it was below the limit of detection by immunoblotting. The intracellular accumulation of adriamycin in ACHN cells was enhanced by verapamil, cyclosporin A and medroxyprogesterone acetate. Therefore, this study has demonstrated that low-level expression of P-glycoprotein detectable only by RT-PCR and flow cytometry plays a significant role in reducing the intracellular concentration of antitumor agents and thus contributes to the multidrug-resistant phenotype of ACHN cells.

Key words: Renal adenocarcinoma — ACHN — P-Glycoprotein — Reverse transcriptase-polymerase chain reaction — Flow cytometry

Despite the multitude of chemotherapeutic agents available, human renal adenocarcinomas do not respond adequately to cancer chemotherapy.[1] The lack of reliable therapeutic alternatives results in a poor prognosis for patients with disseminated disease. This outcome is mainly due to intrinsic multidrug resistance (MDR), which is conferred by the expression of P-glycoprotein (PGP).[2] Several human renal adenocarcinoma cell lines have been established and the majority exhibit PGP expression.[3] The ACHN cell line was established from renal cell adenocarcinoma by Borden *et al.*,[4] and has been accepted as an established cell line with uniform morphology (CRL 1611) by the American Type Culture Collection (Rockville, MD). However, in ACHN human renal carcinoma cells, which are also resistant to a variety of antitumor agents, PGP-expression has not been demonstrated by immunohistochemical staining.[5, 6] Mechanisms of MDR other than the expression of PGP have been postulated to be responsible for intrinsic MDR. In this study, we used a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR)-based assay and a flow cytometric analysis to detect PGP mRNA and the protein in the ACHN cells, in order to assess whether a low-level expression of PGP may have a significant role in creating cells resistant to antitumor agents.

MATERIALS AND METHODS

Cells and cell culture ACHN human renal adenocarcinoma cell line was obtained from the American Type Culture Collection. Cells were grown in monolayer in MEM (Nissui Seiyaku Co., Ltd., Tokyo) containing 10% fetal calf serum and kanamycin. Cultures were grown in 5% CO_2 under 100% humidity at 37°C and the medium was renewed daily from day 7 after seeding.

Drugs and chemicals The drugs were from the indicated sources: adriamycin (ADM), 5-fluorouracil (5-FU) and mitomycin C (MMC) and medroxyprogesterone acetate (MPA) from Kyowa Hakko Co., Ltd., Tokyo; etoposide (VP-16) and cisplatin (CDDP) from Nihon Kayaku Co., Ltd., Tokyo; vinblastine sulfate (VLB) from Shionogi Co., Ltd., Tokyo; verapamil hydrochloride from Eisai Co., Ltd., Tokyo; cyclosporin A (CYA) from Sandoz, Basel, Switzerland; SN-38 from Yakult, Tokyo.

Detection of PGP expression by Western blot hybridization Immunoblot analysis with an antibody specific for PGP was performed to detect PGP.[7] The bovine adrenal gland was used as a positive control for PGP. The homogenized gland and ACHN cells in the exponential growth phase (10^7 cells per flask) were extracted in a solution of 50 m*M* Tris-HCl, pH 8.0 and 1% NP-40. The fractions were centrifuged at 1,500*g* for 5 min and 100 μg aliquots of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel. Protein molecular weight standards were run in parallel in an adjacent lane, and the proteins were visualized by Coomassie blue staining. The proteins were transferred to nitrocellulose with an electroblotting system. The nitrocellulose membrane was blocked with blocking buffer (0.01 *M* phosphate-buffered saline, pH 7.2 [PBS] containing 3% bovine serum albumin) at 4°C overnight. Then the membrane was incubated with 1 μg/ml of C-219 anti-PGP antibody (Centocor Inc., Malvern, PA) in fresh blocking buffer for 1 h at room temperature. After a 15-min wash with washing buffer (PBS containing 0.05% Tween 20), the membrane was incubated with peroxidase-conjugated

rabbit anti-mouse IgG (DAKO, Copenhagen, Denmark) in blocking buffer for 1 h at room temperature. After a 15-min wash with washing buffer, the membrane was incubated in ECL solution (Amersham International plc, Buckinghamshire, UK) for 1 min at room temperature. Finally, the membrane was exposed to Polaroid 667 film.

Detection of PGP mRNA expression by RT-PCR-based assay The mRNA was isolated from ACHN cells by use of a Quick Prep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. The mRNA from the final elution was precipitated with ethanol, dried, and resuspended in 20 μl of 10 m*M* Tris-HCl, pH 7.4, containing 1 m*M* EDTA. The concentration of mRNA was determined by spectophotometry and 0.2 μg of mRNA was used for RT-PCR to detect PGP mRNA and β-actin mRNA. Synthesis of cDNA from the isolated mRNA was performed with a First-Strand cDNA Synthesis Kit (Pharmacia) including Moloney murine leukemia virus reverse transcriptase and pd$(N)_6$ primer in a final volume of 15 μl. Two oligonucleotides used as PCR primers for detecting PGP expression were designed, based on the published sequence of the PGP gene[8]: PGP-A_1, (GGATGGATCTTGAAGGGGA) (nucleotides 423–441, PGP cDNA sequence) and PGP-B, (CCTCCAGATTCATGAAGAACCC) (nucleotides 728–749). The PCR with PGP-specific primers was expected to produce a DNA fragment of 327 bp. The integrity of mRNA isolated from the experimental samples was checked by RT-PCR with primers for human β-actin. The primers for β-actin[9] were 5′-ACAATGAGCTGCGTGTGGCT-3′ (β-A_1) and 5′-TCTCCTTAATGTCACGCACGA-3′ (β-B_1) as described by Ozaki[10] and produced a DNA fragment of 372 bp. A PCR mixture consisted of 10 μl of 10×PCR buffer (500 m*M* KCl-100 m*M* Tris-HCl, pH 8.3-15 m*M* $MgCl_2$-0.01% gelatin), 1 μl of deoxynucleotide triphosphate mixture (20 m*M* each of dATP, dCTP, dGTP, and dTTP), 5 μl of each oligonucleotide primer (4 μM), 0.5 μl of AmpliTaq DNA polymerase (5 units/μl) (Perkin Elmer Cetus, Norwalk, CT), one-half of the cDNA synthesis solution (7.5 μl) including 0.1 μg of mRNA, and 71 μl of distilled water. Finally, 100 μl of paraffin oil was added to prevent evaporation. Thirty incubation cycles (1 min at 95°C, 30 s at 55°C and 30 s at 72°C) were performed. PCR products were electrophoresed on 3% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) with *Hae* III-digested ϕ×174 DNA molecular weight standards and visualized by ethidium bromide staining. Negative control reactions without additional mRNA were used. To minimize contamination, the sample preparations and RT-PCR were performed with the precautionary procedures suggested by Kwok.[11]

Detection of PGP expression by flow cytometry For flow cytometric determination of PGP, monoclonal antibodies (C-219 and JSB-1; Funakoshi Co., Ltd., Tokyo) and isotype control mouse monoclonal immunoglobulins (IgG2a [DAKO] for C-219 and IgG1 [DAKO] for JSB-1) were used.[12, 13] Cells in the exponential growth phase were washed and resuspended in PBS. Cell suspensions were fixed in 100% methanol at −20°C for 15 min, washed with 0.05% Tween 20 in PBS (TPBS), and incubated with C-219 (1.0 μg/ml), JSB-1 (1.0 μg/ml) or an isotype control (IgG2a, 1.0 μg/ml; IgG1, 1.0 μg/ml) at 4°C for 30 min. After incubation, cells were washed with ice-cold TPBS, then incubated with 1.0 μg/ml FITC-labeled goat anti-mouse antibody (MONOSAN, Uden, The Netherlands) for 30 min, passed through a 50 μm nylon mesh and resuspended. Using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), cells were excited with a 488 nm argon ion laser beam and green fluorescence (FITC) was collected with a combination of a 560 dichroic mirror and 530 BP filters. From the FITC fluorescence histograms based on 10,000 gated cells, the mean channel numbers were calculated as a measure of the expression of PGP.

Uptake of ADM The intracellular ADM-uptake experiment was performed by flow cytometric measurements.[14, 15] ACHN cells in the exponential growth phase (10^6 cells per flask) were used in this study. The medium was removed and fresh medium containing 10 μg/ml ADM was added with or without a PGP-inhibitor which showed no cytotoxicity in ACHN cells. The cells in the medium were incubated for 30 min, 1 h, and 2 h at 37°C. Following exposure to the drugs, the medium was removed and the monolayer was washed twice with PBS. Then the cells were trypsinized. The cell suspensions were filtered on a 50 μm nylon mesh and the cells were resuspended in PBS to a concentration of 5×10^5 cells/ml. Using a FACScan flow cytometer, the cells were excited with a 488 nm argon ion laser beam and fluorescence intensity between 530 and 640 nm was evaluated. From the fluorescence histograms based on 10,000 gated cells, the mean channel numbers were calculated as a measure of the intracellular accumulation of ADM. For this experiment, the cytotoxicity of PGP-inhibitors was determined by bioluminescence assay for ATP.[16, 17] Exponentially growing cells were trypsinized, centrifuged, and resuspended in fresh medium. The cell suspension was aliquoted into wells at a density of 10^4 cells per well and each PGP-inhibitor was added in triplicate at appropriate concentrations in volumes of 1 ml. Cultures were incubated at 37°C in humidified 5% CO_2 for 3 days. After continuous exposure to the PGP-inhibitor, the medium was removed and 0.2 ml of ATP-releasing agent (Labo-Science Co., Ltd., Tokyo) was added to each sample. After addition of luciferin and luciferase, a TD-4000 lumiphotometer (Labo-Science) was used for quantitation of ATP levels. Dose-response curves were ob-

tained by calculating the percentage of surviving cells, which was expressed as a percentage of the mean ATP level of the control cells incubated without the agents. The maximum concentration of PGP-inhibitor which showed no cytotoxicity to the ACHN cells was determined (verapamil 2.5 μg/ml, CYA 0.5 μg/ml and MPA 1.0 μg/ml) from the dose-response curves.

RESULTS

P-Glycoprotein expression detected by Western blot hybridization The immunoblotting analysis is shown in Fig. 1. The protein obtained from bovine adrenal gland as a positive control gave a strong signal for PGP with a molecular weight of 170 kDa. However, no signal was detected in the lane on which the fraction extracted from the ACHN cells (100 μg of protein/lane) was loaded.

PGP mRNA expression detected by RT-PCR Detection of PGP mRNA by RT-PCR from ACHN cells is shown in Fig. 2. Various concentrations of mRNA prepared from the ACHN cells were loaded using primers specific for PGP mRNA or for β-actin. Bands of PGP and β-actin were detected at quantities as low as 100 pg of mRNA prepared from the ACHN cells.

P-Glycoprotein expression detected by flow cytometry Fig. 3 illustrates the frequency histograms of fluorescence intensity for ACHN cells after incubation with PGP-specific antibodies (C-219 and JSB-1) or isotype control immunoglobulins. The ACHN cells expressed PGP with nearly one logarithm difference in peak channel values between the isotype control and the sample treated with C-219 or JSB-1 antibody. The mean channel values obtained from each histogram were higher in the cells treated with C-219 antibody than in those treated with JSB-1 antibody.

Intracellular ADM uptake The intracellular ADM accumulation in ACHN cells exposed to 10 μg/ml of ADM with or without modulators is shown in Fig. 4. An initial rapid uptake of the drug was observed for the first 30-min period, followed by a slow uptake that continued up to 120 min or reached a plateau. The addition of non-cytotoxic concentrations of verapamil (2.5 μg/ml), CYA (0.5 μg/ml) or MPA (1.0 μg/ml) enhanced the initial uptake of the drug and steadily increased the intracellular ADM concentration in ACHN cells. The intracellular ADM accumulation over 120 min was increased 1.8 times, 1.9 times, and 2.6 times by verapamil, CYA, and MPA, respectively.

DISCUSSION

To devise therapeutic strategies for successful cancer chemotherapy, it is necessary to understand the molecular mechanisms underlying the development of resistance to antitumor agents. Although the mechanisms include variations of glutathione and metallothionein levels, topoisomerase activities, DNA repair capacity and so on, PGP is one of the most important factors in the mechanism of MDR. Many attempts have been made to detect

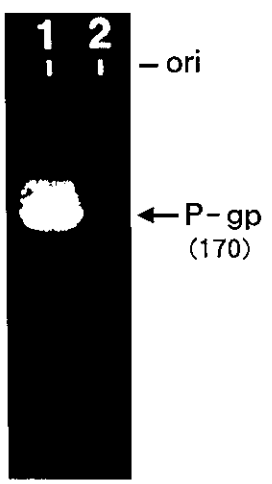

Fig. 1. Western blot hybridization analysis of PGP in the ACHN cells. The protein obtained from the bovine adrenal gland was used as the positive control (lane 1). Lane 2 contains the fraction extracted from ACHN cells (100 μg of protein/lane). The protein from the bovine adrenal gland gave a strong signal for PGP with a molecular weight of 170 kDa, but no signal was detected in lane 2.

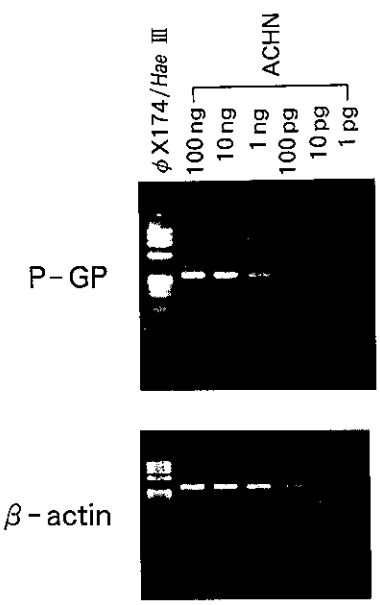

Fig. 2. Detection of PGP mRNA from the ACHN cells. The amplified DNA fragments for PGP and β-actin were both detected in quantities as low as 100 pg of mRNA.

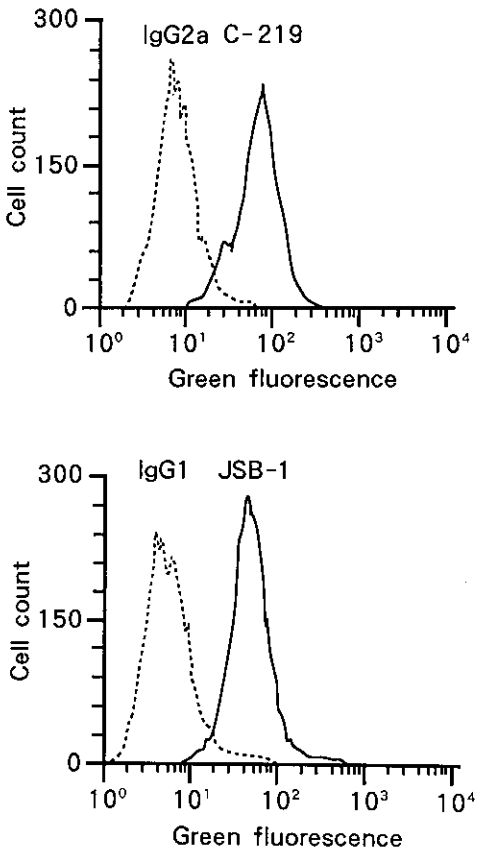

Fig. 3. Expression of PGP in the ACHN cells analyzed by flow cytometry. A solid line represents a fluorescence histogram of PGP-specific antibody (C-219 or JSB-1) and a dotted line represents a fluorescence histogram of control antibody (IgG2a antibody for C-219 or IgG1 antibody for JSB-1). The ACHN cells express PGP with nearly one logarithm difference in peak channel values between the isotype control and the sample treated with C-219 or JSB-1 antibody. The mean channel values obtained from each histogram were higher in the cells treated with C-219 antibody than in those treated with JSB-1 antibody.

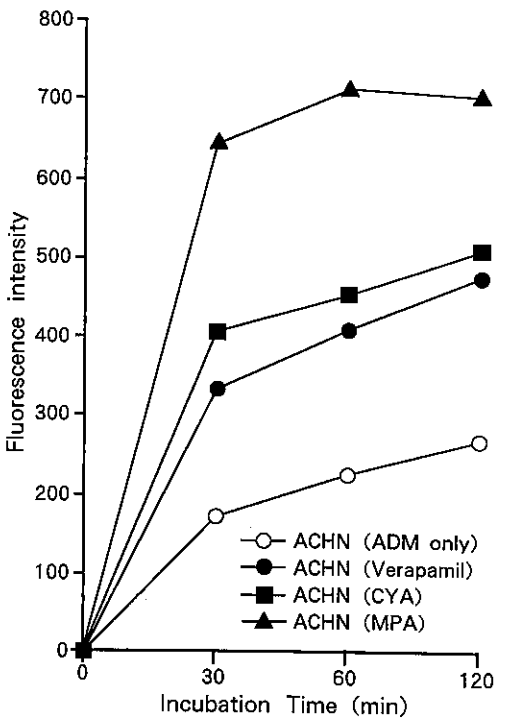

Fig. 4. The intracellular ADM accumulation in ACHN cells. ACHN cells were incubated in media containing 10 μg/ml of ADM in the absence (○) or presence of 2.5 μg/ml of verapamil (●), 0.5 μg/ml of CYA (■), or 0.1 μg/ml of MPA (▲). An initial rapid uptake of the drug was observed for the first 30-min period, followed by a slow increase that continued up to 120 min or reached a plateau. The addition of verapamil, CYA or MPA enhanced the initial uptake of the drug and steadily increased the intracellular ADM concentration in the cell line. The intracellular ADM accumulation over 120 min was enhanced 1.8 times, 1.9 times, and 2.6 times by verapamil, CYA, and MPA, respectively.

PGP in cell lines and clinical specimens by means of several methods. However, discrepancies between MDR phenotype and PGP expression have been reported.[5,6] ACHN cells, which were established from renal adenocarcinoma, are resistant to various antitumor drugs, but PGP expression has not been detected by immunohistochemical staining in the cells.[5,6] These cells have been classified as PGP-negative with MDR phenotype. In this study, however, PGP was detected at the mRNA and protein levels in ACHN cells by highly sensitive RT-PCR and flow cytometric analysis, though it was below the limit of detection by immunoblotting analysis. This study also demonstrated that the intracellular accumulation of ADM in ACHN cells was enhanced by verapamil, cyclosporin A and medroxyprogesterone acetate. These compounds have been reported to reverse multidrug resistance *in vitro* and *in vivo* by competitively inhibiting the binding of antitumor agents to PGP in the resistant tumor cells and increasing the intracellular accumulation of antitumor agents.[18–20] Previous studies have found that the sensitivities of ACHN cells to antitumor agents are enhanced by MDR-reversing agents.[5,6] These findings indicate that ACHN cells have a low expression level of PGP, which cannot be detected by immunohistochemical staining or immunoblotting, but only by highly sensitive RT-PCR or flow cytometric analysis, and that the low-level expression of PGP detectable by these assays has a significant role in reducing the intracellular accumulation of antitumor agents and thus contributes to the MDR phenotype of ACHN cells.

Certainly we are aware that limitations of this study include the absence of any assessment of other mechanisms of MDR in ACHN cells and insufficient quantitative information about the association between PGP level and MDR level. However, this study provides sufficient data to indicate that MDR of ACHN cells is conferred in part by the expression of PGP, as is the case in other renal adenocarcinoma cell lines and clinical renal cancers,[3] and we suggest that, in order to understand the mechanisms of MDR in tumor cells such as ACHN cells, highly sensitive methods that are capable of detecting the low-level expression of PGP demonstrated here are required.

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