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DETECTION OF SOLUBLE P-GLYCOPROTEIN IN CULTURE MEDIA AND EXTRACELLULAR FLUIDS

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SUMMARY Multidrug resistance (MDR) is a unique phenomenon in cancer patients and is commonly associated with an overexpression of the human MDR gene *mdr1*, which encodes an energy-dependent Mr 180 kDa membrane bound protein, known as P-glycoprotein. P-glycoprotein serves as a membrane efflux to pump the drugs out of the cancer cells. Western blot analysis, using a newly generated monoclonal antibody F4 which recognizes specifically an extracellular epitope of human MDR1 P-glycoprotein, reveals that soluble P-glycoprotein is detected in the cultured media of viable adriamycin-resistant human ovarian carcinoma 2780^{AD} cells, whereas those of the drug-sensitive parent A2780 cells contain no detectable level of soluble P-glycoprotein. Soluble P-glycoprotein also is detected in extracellular fluids of cancer patients, such as malignant ascites and serum, and is not detectable in serum samples of normal healthy individuals. The Mr of soluble P-glycoprotein is the same as that of membrane bound P-glycoprotein. The presence of soluble P-glycoprotein in extracellular fluids may provide the basis for its use as a quantitative parameter of MDR and as a means to lessen or reverse MDR.

The development of drug resistance is a common yet unique phenomenon in cancer patients (1-3). There are basically two classes of drug resistance. The first class represents resistance to a single agent or a closely related group of agents. The other class is collectively referred to as multidrug resistance (MDR), which describes a phenotype whose predominant feature is resistance to a series of multiple agents that are unrelated to each other in structure and mode of action; *e.g.*, anthracyclines, antimitotics, antibiotics, *Vinca* alkaloids, epipodophyllotoxins, digitalis (1-10). One common feature these drugs share is that they are all moderately soluble both in water and lipid. The clinical cause of MDR *in vivo* remains unclear. Investigation using human and animal cell lines *in vitro* has identified the association of MDR with an overexpression of several proteins (11,12), the best known of which is an energy-dependent Mr 180 kDa transmembrane protein, known as P-glycoprotein. P-glycoprotein initially was thought to function as a *permeability* barrier that prevented cytotoxic agents from entering the cells. However, it has been determined

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Abbreviations: MDR, multidrug resistance; McAb, monoclonal antibody; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence.

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that one biological function of P-glycoprotein is to serve as a drug efflux pump; *i.e.*, to transport chemotherapeutic agents out of cancer cells (1-12).

P-glycoprotein is encoded by a small family of genes (13-17). Two human genes (*mdr1, mdr3*), three mouse genes (*mdr1, mdr2, mdr3*), and three hamster genes (*pgp1, pgp2, pgp3*) have been identified and cloned. The human MDR genes show 80% nucleotide homology. Human MDR3 and mouse MDR3 are similar. Biologically, only the human MDR1 encoded P-glycoprotein is active in multidrug resistant cells serving as a membrane efflux to pump the drugs out of the cells. Genetic and molecular biological investigation has revealed that MDR1 encoded prototype P-glycoprotein consists of twelve transmembrane domains and two cytoplasmic ATP binding sites, with two highly symmetrical peptide halves. Biochemically, P-glycoprotein is found in plasma membrane enriched fraction, is glycosylated, exhibits ATPase activity, and binds photoactivated ATP and drug analogs (4-9). Recent experiments transfecting the cDNA sequence of the human *mdr1* gene into drug sensitive host cells, resulting in subsequent expression of the MDR, further support the crucial role played by P-glycoprotein in MDR (18,19).

P-glycoprotein can be determined by a wide variety of methods which have been developed over the years: Northern blot, Southern blot, Western blot, polymerase chain reaction, *in situ* hybridization, immunohistochemistry, flow cytometry, labeled drug (doxorubicin, cytochalasin) accumulation, and MTT test-cell viability (5,7,9,11,12). As noted, all of these methods require tumor tissue specimens and are based upon the very fact that P-glycoprotein is a plasma membrane bound component (1). During the course of our investigation on the expression of P-glycoprotein by human tumors, as monitored by Western blot with a newly generated monoclonal antibody (McAb) F4 which recognizes an extracellular epitope (20), we have detected the presence of soluble P-glycoprotein exhibiting a same Mr 180 kDa in cultured media collected from fresh and viable adriamycin-resistant human ovarian carcinoma cell line 2780^{AD}, and the absence of soluble P-glycoprotein in parent adriamycin-sensitive cell line A2780. Additionally, extracellular fluids obtained from cancer patients, such as malignant ascites and serum, were found to contain soluble P-glycoprotein, whereas those from normal healthy individuals were found to express no detectable level of P-glycoprotein. These results describe for the first time the presence of soluble P-glycoprotein in cancer patients. Although the exact pathophysiological nature of soluble P-glycoprotein in extracellular fluids remains to be determined, it may play a role as a quantitative parameter for MDR and may serve as a means for clinical manipulation of MDR reversal.

MATERIALS AND METHODS

Cell line and culture

Human ovarian carcinoma cell line A2780 sensitive *in vitro* to adriamycin, and its adriamycin resistant variant 2780^{AD} were maintained *in* RPMI 1640 *with* 10% fetal calf serum (FCS) (20). In addition, 2780^{AD} cells were maintained in culture containing adriamycin at 1 μg/ml. Adriamycin (doxorubicin) was kindly provided by Adria Laboratories (Columbus, OH). Cell culture media and reagents were purchased from GIBCO (Grand Island, NY).

Murine McAb F4 was generated as described (20). Murine McAb JSB-1 and C219 were purchased from Sigmet (Dedham, MA) and Centocor (Malvern, PA), respectively.

Drug-sensitive human melanoma parent cell line SW1573, SW1573 MDR1-transfected cell line, and MDR3 transgenic mouse ear fibroblast cell line V01V01 were grown in complete

Dulbecco's modified Eagle's medium, *i.e.*, supplemented with 2 mM L-glutamine, penicillin 50 units/ml, streptomycin 50 µg/ml, and 10% (v/v) heat-inactivated FCS, in the presence of 5% CO_2 at 37 C. Drug-resistant clones were cultured under vincristine selection (10 mM) (18,19).

Human extracellular fluid specimens

Ascitic fluids were obtained from patients with breast cancer or ovary cancer (21,22). The presence of malignant cells in the ascites specimens was confirmed by cytological examination (21,22). Blood samples from patients with histologically confirmed breast cancer, ovarian cancer, or prostate cancer were obtained from our Institute (23,24). Seminal plasma specimens were obtained from vasectomized men at their routine clinic visit (25). All specimens were stored at -70 C until used.

Immunoprecipitation and Western blotting

Cell lysate, crude plasma membrane preparations, or concentrated cultured media, ascites, serum, were incubated with primary monoclonal antibody at 4 C for 3 hr, or overnight (20). Appropriate controls were also performed. Immunobeads (BioRad, Hercules, CA) coated with rabbit anti-mouse immunoglobulin, in excess amount, were added to the mixture and incubated at 4 C for an additional 3 hr. The precipitated proteins were dissolved in SDS-PAGE sample treatment buffer and the dissociated proteins were subjected to 7.5% SDS-PAGE under reducing conditions. Proteins were transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked with 3% nonfat milk and 2% BSA in PBS, and incubated with diluted specific antibody at room temperature for 1 hr. After washing with PBS, the nitrocellulose membrane was incubated with 1:3000 dilution of peroxidase conjugated goat anti-mouse immunoglobulin (Amersham, Arlington Heights, IL) in blocking buffer containing 0.1% Tween-20 at room temperature for 1 hr (26,27). Following extensive washing, the immobilized proteins were detected by an enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Immunoprecipitation of plasma membrane P-glycoprotein

To demonstrate further the reactivity of McAb F4 with the multidrug resistant membrane bound P-glycoprotein, crude membrane proteins prepared from adriamycin-resistant human ovarian carcinoma 2780^{AD} cells and adriamycin-sensitive A2780 cells were incubated with McAb F4, and then immunoprecipitated by rabbit anti-mouse immunoglobulin in solid phase. Control precipitation was performed with a mixture of crude membrane proteins prepared from drug-resistant 2780^{AD} cells and normal mouse immunoglobulin. The precipitated proteins were subjected to SDS-PAGE and analyzed by immunoblot with P-glycoprotein specific McAb C219 (28). Figure 1 reveals that McAb F4 precipitates P-glycoprotein of crude membrane preparation from 2780^{AD} cells, and that McAb F4 precipitates no P-glycoprotein of crude membrane preparation from A2780 cells. The broad apparently overloaded band detected at 45-60 kDa in all lanes represents that of dissociated heavy chain mouse IgG; that of light chain (Mr 25 kDa) is not detectable under the present experimental condition.

Identical experiments were performed with another P-glycoprotein specific McAb JSB-1 (29). The F4 immunoprecipitated plasma membrane protein also was shown to react with McAb JSB-1 (data not shown). These results conclude that McAb F4 recognizes specifically P-glycoprotein.

Recognition of human MDR1 P-glycoprotein

In order to ascertain that the reactivity of the McAb F4 is directed specifically against human MDR1 gene encoded P-glycoprotein, and not against mouse MDR3 gene encoded P-glycoprotein, a series of experiments utilizing human melanoma transfectant cell line SW1573 containing the human MDR1 cDNA were performed, along with the drug sensitive parent cell line SW1573 and a transgenic mouse ear fibroblast line V01V01 containing mouse MDR3 cDNA as the negative control. McAb JSB-1 and C219 were used as the control antibodies.

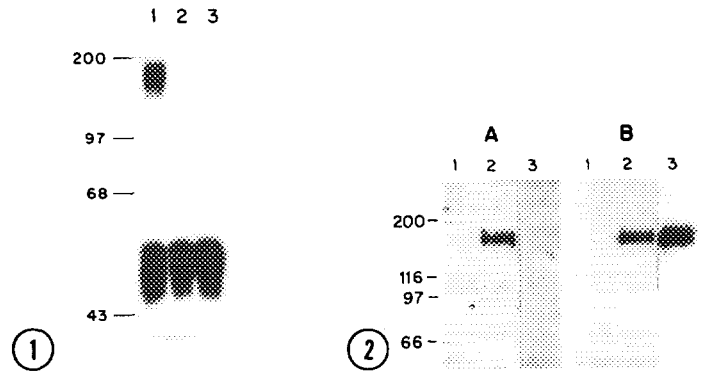

Fig. 1. Reactivity of McAb F4 with P-glycoprotein from plasma membrane of drug-resistant 2780^{AD} cells . Crude membrane proteins (800 μg) each from adriamycin-resistant human ovarian carcinoma 2780^{AD} cells (lane *1*) and drug sensitive A2780 cells (lane *2*) were incubated with McAb F4 (80 μg) at 4°C overnight. Control precipitation was performed with the mixture of crude membrane proteins from 2780^{AD} cells and normal mouse immunoglobulin (lane *3*). Immunobeads (2.5 mg) coated with rabbit anti-mouse immunoglobulin were added to the mixture and incubated. The precipitated proteins were dissociated in 200 μl of SDS-PAGE sample treatment buffer. Fifty μl of samples containing the dissociated proteins was subjected to SDS-PAGE in reducing condition, transferred onto nitrocellulose membrane, immunoblotted with P-glycoprotein reactive McAb C219 (5 μg/ml), and detected by ECL. P-glycoprotein is indicated at 180 kDa on lane 1. Molecular weight markers in kDa are shown at the positions on the left.

Fig. 2. Recognition of human MDR1 P-glycoprotein by McAb F4. Total cell lysates were prepared and DNA was sheared by sonication. Proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted by primary antibody McAb F4 and C219, respectively. Secondary antibody used was peroxidase conjugated goat anti-mouse IgG, and the immunoblot was detected by ECL (Amersham). Panel ***A:*** blotting with F4 (10 μg/ml). Panel ***B:*** blotting with C219(4 μg/ml). Lane *1*: SW1573 drug sensitive cell line; lane *2*: SW1573 MDR1-transfected cell line expressing human MDR1 P-glycoprotein; lane *3:* MDR3 transgenic mouse ear fibroblast line V01V01 expressing MDR3 P-glycoprotein. Total cell lysate, 10 μg protein, was used in each lane. Molecular weight markers in kDa are shown at the positions on the left.

Cell lysate preparations were made from these three cell lines and were subjected to SDS-PAGE and Western blot analysis. As shown in Figure 2, comparing the results obtained from control McAb C219 which is known to react with both human MDR1 P-glycoprotein and mouse MDR3 P-glycoprotein (panel B) (18,28), McAb F4 recognizes specifically the human MDR1 P-glycoprotein (panel A, lane 2). Data not shown include that the reactivity and sensitivity of McAb F4 Western blot are very similar to that of McAb JSB-1, which is known to recognize specifically human MDR1 P-glycoprotein (18,28). Additionally, McAb F4 does not appear to recognize the mouse *mdr1a* and *mdr1b* P-glycoprotein (30) . Therefore, these results demonstrate unequivocally that McAb F4 recognizes specifically the human MDR1 P-glycoprotein.

Detection of soluble P-glycoprotein in cultured media of viable cells

The demonstration of the presence of soluble P-glycoprotein is a significant observation of this report. To demonstrate the presence of soluble P-glycoprotein, rather than as an end-product of degradation of the cells; *e.g.*, cell debris, cultured media collected from fresh and viable 2780^{AD} cells were examined for the presence of soluble P-glycoprotein. Concentrated culture media collected from fresh and viable 2780^{AD} cells and from A2780 cells were immunoprecipitated with McAb F4. The precipitated immunocomplexes were examined by SDS-PAGE and

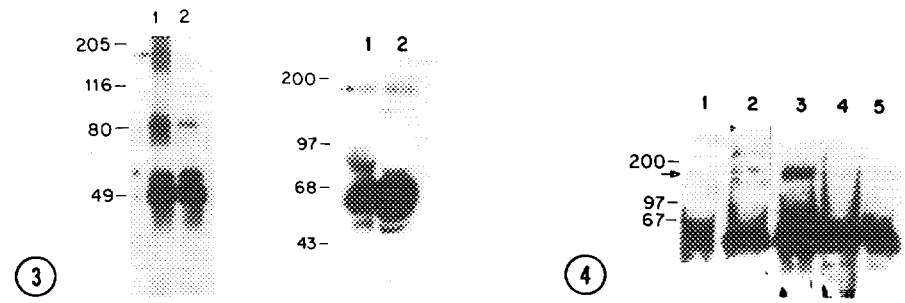

Fig. 3. Demonstration of soluble P-glycoprotein in cultured media of drug resistant 2780^{AD} cells and in malignant ascites. Concentrated cultured media from fresh and viable 2780^{AD} cells (1.6 ml, lane *1*) and fresh and viable drug-sensitive A2780 cells (1.6 ml, lane *2*) were incubated with 100 μg each of McAb F4. Immunobeads (2.5 mg) coated with rabbit anti-mouse immunoglobulin were added to the mixture, and the precipitated proteins were dissolved in 200 μl of SDS-PAGE sample treatment buffer. Fifty μl of sample containing the dissociated proteins was subjected to SDS-PAGE in reducing condition, transferred to nitrocellulose membrane, and immunoblotted with MDR1 P-glycoprotein McAb JSB-1 (5 μg/ml) and detected by ECL procedure (***LEFT***). Concentrated 2780AD (18x) cultured media from viable adriamycin-resistant human ovarian carcinoma 2780^{AD} cells (1.2 ml, lane *1*) and malignant ascites of breast cancer (8 ml, lane *2*) were incubated with 200 μg and 500 μg of McAb F4, respectively, and treated as described above (***RIGHT***). P-glycoprotein is indicated by arrow. Molecular weight markers in kDa are shown at the position on the left.

Fig. 4. Detection of soluble P-glycoprotein in malignant ascites by McAb F4. Malignant ascites (1 ml) was centrifuged. Supernatant was collected and added with 50 μg each of McAb F4, MRK16, C219, JSB-1, and control normal mouse IgG and incubated. After precipitation by immunobeads (4 mg) coated with rabbit anti-mouse immunoglobulin, the precipitated proteins were dissolved in 50 μl of SDS-PAGE sample treatment buffer and subjected to SDS-PAGE in reducing condition. Western blot was performed, Lanes ***#1, 2, 3, 4*** and ***5*** were precipitated by normal mouse IgG, MRK16, F4, C219, and JSB-1 and then blotted by F4 (4 μg/ml), F4 (4 μg/ml), F4 (4 μg/ml), C219 (1 μg/ml) and JSB-1 (5 μg/ml), respectively. Immunoblots were detected by ECL procedure. P-glycoprotein is indicated by arrow. Molecular weight markers in kDa are shown at the positions on the left.

immunoblot analysis using McAb JSB-1. As shown in Figure 3, soluble P-glycoprotein is detected in the cultured media of adriamycin-resistant 2780^{AD} cells, whereas the cultured media of adriamycin-sensitive A2780 cells contain no detectable soluble P-glycoprotein. One notable observation is that molecular size of soluble P-glycoprotein, approximately 180 kDa, is the same as that of membrane bound P-glycoprotein. The protein bands(s) detected in the cultured media of both 2780^{AD} and A2780 cells at the range of 80 kDa is not identified.

These results are consistent with those presented in Figure 1. In combination with the results as shown in Figure 1, these experiments clearly indicate that soluble P-glycoprotein detected in the spent cultured media collected from the adriamycin-resistant 2780^{AD} cells is the result of an overexpression of adriamycin-resistant cell membrane bound P-glycoprotein.

Detection of soluble P-glycoprotein in extracellular fluids of cancer patients

Figure 3 also reveals the presence of soluble P-glycoprotein in the specimen of malignant ascites obtained from a breast cancer patient as detected by McAb F4. Further experiments revealed that 7 out of 10 malignant ascites specimens obtained from cancer patients were shown to contain soluble P-glycoprotein, as demonstrated by the technique of McAb F4 immunoprecipitation and immunoblotting as described. Additionally, 2 of 9 cancer serum

specimens and none of 5 normal serum samples were shown to contain soluble P-glycoprotein. No soluble P-glycoprotein was detected in 4 human seminal plasma specimens examined.

In order to determine that the recognition of an epitope expressed by soluble P-glycoprotein is a feature of McAb F4, the results of immunoreactivity, precipitation and Western blot analysis by McAb F4 were compared with those obtained from the most commonly known McAb directed against P-glycoprotein; *e.g.*, C219 (28), MRK16 (31), and JSB-1 (29).

In this series of experiments, ascites specimens obtained from cancer patients were used instead of the *in vitro* prepared culture media. The ascites was precleared by centrifugation. Supernatant was collected and reacted with McAb F4, C219, JSB-1, MRK16, and control normal mouse IgG. Thereafter, solid phase coated rabbit anti-mouse immunoglobulin was added to each specimen to precipitate McAb F4- P-glycoprotein immune complexes. The precipitated immune complexes were analyzed by Western blotting using McAb F4, C219, and JSB-1, respectively. Shown in Figure 4 are the results obtained from one malignant ascites from a breast cancer. Ascites was first precipitated by normal mouse IgG, MRK16, F4, C219, and JSB-1, and then blotted by F4, F4, F4, C219, and JSB-1, respectively (*i.e.*,lane #1, normal mouse IgG→F4; lane 2, MRK16→F4; lane #3, F4→F4; lane #4, C219→C219; lane #5, JSB-1→JSB-1). As shown, the only positive result was detected on lane #3, *i.e.*, only McAb F4, not MRK16, or JSB-1, is able to bind and precipitate soluble P-glyco-protein from ascites specimen. Normal mouse IgG served as the negative control. The result from C219 is equivocable.

Except the Mr, the comparison between soluble P-glycoprotein and membrane bound-P-glycoprotein was not performed at this investigation, as soluble P-glycoprotein has not been isolated in quantity large enough for biochemical characterization, such as the measurement of ATPase (4).

The exact pathophysiological role of soluble P-glycoprotein is not known at present. Cell culture data indicate that the presence or an increased level of soluble P-glycoprotein appears to derive from the overexpression of drug-resistant cell membrane P-glycoprotein. The overexpression of membrane P-glycoprotein could lead to the secretion and accumulation of soluble P-glycoprotein in extracellular fluids surrounding the tumor cells and in the circulation (serum). Should soluble P-glycoprotein retain the ability to bind chemotherapeutic agents, its presence or in excess amount is likely to increase the drug-resistance of the patient. On the other hand, *ex vivo* removal of soluble P-glycoprotein could lessen or reverse MDR, and may represent a new strategy for chemotherapy. Additionally, quantitation of soluble P- glycoprotein in extracellular fluids could represent a parameter for MDR and provide an objective measure for the efficacy of cancer chemotherapy.

It is noted that at the completion of this investigation, an abstract appeared most recently reporting the detection of P-glycoprotein in serum samples of breast cancer patients with an enzyme immunoassay (32).

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