Ph.D. Thesis

Quantitative and functional analysis of MDR1/P-glycoprotein from human tumors

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2007

1. Introduction:

Ovarian cancer is the fourth most common gynecological malignancy, but due to its poor outcome the leading cause of mortality among gynecological cancers. The tumor spreads early in the disease to the peritoneal cavity, and the tumor cells often remain confined to the peritoneum and an *en block* resection of the tumor is usually not possible. Therefore in the treatment of the disease surgery should always be followed by chemotherapy except for the earliest stage of the disease. The major problem of chemotherapeutic cancer treatment is the intrinsic or acquired multidrug resistance (MDR) which results in a reduced accumulation of the drug in the cancer tissues.

There are many factors involved in the formation of ascites in malignancies, including cytokines, direct lymphatic blockade, tumor neovascularization, etc. In the case of ovarian cancer, it is a sign of peritoneal implantation/dissemination and a bad prognostic factor. Today, in the first-line treatment of ovarian cancer the gold standard is the platinum based chemotherapy in combination with taxanes. In second and third lines the most commonly used drugs are the topoisomerase inhibitors, taxan derivatives and anthracyclines. To prevent resistance or to decrease the intrinsic or acquired resistance in the clinical practice drugs are often used in combinations. The above mentioned drugs except the platinum based ones are hydrophobic compounds.

In general, multidrug resistance refers to a phenotype, in which a tumor is resistant to a large number of chemotherapeutic drugs. This phenomenon is frequently associated with and caused by the overexpression of an ABC1 transporter, the P-glycoprotein (Pgp). Recent publication refers that around 50% of the chemotherapeutic drugs used in clinical practice are the substrates or modulators of this transporter protein. The Pgp is an ATP-ase with wide substrate spectra which recognizes a number of amphyphyl and lipophyl compounds among them most of the drugs used in chemotherapeutic treatment of ovarian cancer. The transporter is often overexpressed on the surface of the cancer cells, since these cells enjoy selection advantage during chemotherapy. Baekeland et al. reported, that 47% of the patients with ovarian cancer were Pgp positive before chemotherapeutic treatment, and they found the presence of Pgp being a bad prognostic factor. They also showed that a portion of the patients became Pgp positive during chemotherapy, and found positive correlation between the presence of ascites and Pgp positivity.

The recognition and characterization of the multidrug-resistant cells in ovarian cancer samples is a very important clinical parameter. Several kinds of monoclonal and polyclonal antibodies were developed to detect Pgp. Flow cytometry is the most suitable diagnostic cell analysis technique to identify precisely the various subtypes of cells or the presence of expressed proteins in the cell membrane. Up to now several fluorescence assays have been elaborated to detect multidrug sensitive and resistant cells but none of them dealt with cells from ascites of patients with ovarian cancer. In our present work we describe a flow cytometric method that provides tool for the quantitative estimation of the expression with parallel measurement of the function of the Pgp multidrug transporter in tumor cells from ascites of patients with malignant ovarian cancer.

Positron emission tomography is the most frequently used "in vivo, noninvasive" tumor diagnostic method. The most commonly used PET tumor diagnostic radiopharmacon is the ^{18}F-labelled glucose analogue the [^{18}F]fluoro-dezoxy-glucose (^{18}FDG), which accumulates in the tumor cells due to their high metabolic activity. Similarly to the ^{18}FDG, the ^{11}C-choline also accumulates in an increased rate in the malignant tissues. Choline is incorporated in cells through phosphoryl choline synthesis and is integrated in the membrane phospholipids. The malignant transformation of cells is associated with the induction of choline kinase activity, resulting in increased level of phosphoril choline for the synthesis of membrane phospholipids. The ^{99m}Tc-hexakis-2-methoxybuthyl isonitrile (^{99m}Tc-MIBI) is also a well known and widely used SPECT tumor diagnostic radiopharmacon which compound is the substrate of the Pgp pump. This molecule has a single positive charge which accumulates in the mitochondria of the tumor cells. Recent publications attracted the attention to the observation, that different ligands, drugs used in clinical chemotherapy may modify the accumulation of the PET or SPECT tumor diagnostic tracers both in tumor cells and solid tumors, hereby may mask the correct, proper diagnosis.

Paclitaxel and other taxane derivates are widely used chemotherapeutic agents for clinical treatment of various tumors, such as ovarian cancer, breast cancer and non-small cell lung cancer. It has been proved that paclitaxel is the substrate of the Pgp pump. Although intensive research is going on to discover in details the mechanism of action of the drugs used in chemotherapy, the effect of paclitaxel on the accumulation of PET and SPECT tumordiagnostic tracers has not been yet

investigated. The aim of our study was to investigate how paclitaxel modifies the accumulation of the above tracers in Pgp$^+$ and Pgp$^-$ cancer cells. As a model we used a cell line of ovarian cancer origin.

PET is a non invasive medical imaging system suitable for the measurement of the rate of various metabolic activities in living organisms. The knowledge of the exact kinetic parameters of the glucose metabolism makes the clinical tests, made at different places and/or time, comparable. It also provides effective help in establishing a correct diagnosis, in the prognosis of the disease-process and in the estimation of the efficiency of the applied therapy. The quantization of the images (which results in numerical data) can be done using several different tracer-kinetic methods. Among them the most precise is the Phelps model (Phelps et al. 1977, 1979). Other simplified methods are the so called Patlak and SUV methods (Patlak et al. 1983, Woodard et al. 1975).

Several publication deals with the comparison of parameters determined by using different kinetic models, but the systematic comparison and analysis of the above mentioned three most frequently used models have not been done yet. In our laboratory we made the quantitative analyses using the above methods and compared the results of the FDG-PET brain scan of five healthy volunteers.

2. Objectives

1. Elaboration of a noninvasive flow cytometric method for the detection of the expression and function of Pgp in tumor cells derived from ascites of patients with ovarian cancer.

2. To measure the effect of paclitaxel on the accumulation of different tumor-diagnostic tracers (^{18}FDG, ^{11}C-choline, ^{99m}Tc-MIBI) in cancer cells. To determine the Pgp dependent and Pgp independent effects of paclitaxel on the ^{18}FDG, ^{11}C-choline, ^{99m}Tc-MIBI accumulation.

3. Comparative analysis of different kinetic models used for the analyses of FDG-PET brain scans.

3. Materials and methods

Cell culture

In our experiments we used the human adenocarcinoma-derived ovarian cell line and its doxorubicin-selected Pgp expressing counterpart (A2780 and A2780AD, respectively), the Epstein-Barr virus-transformed JY human B-lymphoblast cell line, the NIH 3T3 mouse fibroblast cell line and its human MDR1 gene transfected counterpart, the NIH 3T3 MDR1 G185 cell line.

Cell separation from ascites

The ascites was centrifuged (for 10 min at 800 g) and -following the lysis of red blood cells in standard ammonium chloride lysing solution- the cells were washed in PBS and the pellet was resuspended in PBS containing 5 mM D glucose.

Flow cytometry

Becton Dickinson FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) equipped with two (Ar ion and He-Ne) lasers or a Becton Dickinson FACScan flow cytometer were used to determine fluorescence intensities. The excitation was adjusted to 488 nm while the detection was done at 540 nm in case of R-123 or above 580 nm in case of DNR. The viability and the DNA content of the cells were determined by using propidium iodide (PI). PI was excited at 488 nm and detected above 620 nm.

The detection of the Pgp was done by immunofluorescence technique. The expression of the MDR1 gene was measured on native and formaldehyde fixed samples. The cells were washed in PBS and incubated with anti Pgp antibodies $(10 \mu gml^{-1})$ prepared from the supernatant UIC2 hybridoma cells or with MM615 monoclonal antibody against Pgp (M. Cianfriglia, Instituto Superiore di Sanita, Rome, $8 \mu gml^{-1}$) for 40 min on ice in PBS containing 1% fetal calf serum. As secondary antibodies FITC-RAMIG, Alexa 488 RAMIG or Alexa 647 RAMIG was used (incubation for 40 min on ice). Nonspecific mouse IgG2a antibody (UPC10, Sigma-Aldrich, St. Luis, MO) was used for isotypic control. The ovarian cancer cells were identified by CA125 monoclonal antibody (mouse anti human CA125 IgG1, MCA1914H, Serotec Ltd, Kidlington, Oxford).

Production of the radiopharmacons

The ^{18}FDG and the ^{11}C-choline PET radiotracers were synthesized in the radiochemical laboratory of the University of Debrecen PET Centre. MIBI was purchased from the F.J.Curie Radiobiological Research Institute and labeled with ^{99m}Tc according to the kit instruction in the lab of the Nuclear Medicine Institute of the University of Debrecen.

Radiopharmacon uptake studies

The cells were washed and resuspended in PBS. The samples were preincubated at 36°C for 10 min at a cell concentration of 1×10^{6} ml^{-1} in PBS containing 5 mM D-glucose and other agents (paclitaxel, verapamil, CSA, etc) and 5 µCiml^{-1} ^{18}FDG, or 50 µCiml^{-1} ^{11}C-choline, or 5-10 µCiml^{-1} ^{99m}Tc-MIBI was then added to each sample. After the addition of the radioligands the cells were further incubated at 36°C for the given experimental period of time and the uptake was then terminated by the addition of ice cold PBS. The cells were then washed three times in cold PBS and the radioactivity was measured in a calibrated Canberra Packard gamma counter.

Data analyses

The data were expressed as mean ± SD of at least three independent experiments. Tracer accumulation data were compared with those measured under control conditions using Student's t-test (two-tailed). The level of significance was set at P=0.01 unless otherwise stated.

Comparison of FDG PET quantitative kinetic models

GE 4096 Plus whole body PET camera was used for quantitative accumulation measurements. The experiments were made on 5 healthy volunteers and were approved by the Research Ethical Committee of the University of Debrecen. ^{18}FDG was used as radiotracer. 0,15 mCikg^{-1} bodyweight activity was injected into the left or right cubital vein during 10 s in 5 ml physiological salt solution. The collection of the data started parallel with the injection of the radiopharmacon. In the first six minutes twelve samples were collected (one in every 30 s) then four samples with 1 min incubation, then seven samples with 5 min incubation and the ^{18}FDG activity in the

plasma was determined by a calibrated gamma counter. The glucose metabolic rate of the brain was done by using three compartments model. The analyses were done on VAX 4000 VLC workstation using Image Display and Analysis 6.1 program. The kinetic parameters were determined by the MATLAB program on a Silicon Graphics INDIGO[2] workstation.

On each person 115-130 ROI were examined which covered 22-25 well defined anatomical regions of the brain. The cerebellum*; pons; thalamus; gyrus rectus*; corpus callosum; gyrus cinguli*; slices from the cortical regions of the frontal lobe*, temporal lobe*, parietal lobe*, occipital lobe*; capsula interna*; nucleus lentiformis*; nucl. caudatus*; and cuneus* were examined. The area marked with asterix contained two symmetrical (left and right) regions. The three models were compared by linear correlation analyses.

4. Results and discussion

4.1 Expression and function of Pgp protein derived from ascites of patient with ovarian cancer

Expression of Pgp protein

Pgp was detected by means of indirect immunofluorescence, using monoclonal antibodies (UIC2 and/or MM615) recognizing the extracellular epitope of the protein. As negative control human peripheral blood lymphocytes (HPBL) and JY human lymphoid cell line were used. The mean fluorescence intensity of the samples was normalized to the isotypic control to generate a signal to background ratio, R, which varied between 2.0 and 18 for the MM6.15 (mean±SD 38.9±20.7, n=35) and between 1.5 and 15 for the UIC2 (mean±SD 42±16.7, n=11). A very strong correlation was found between the R values of parallel samples labeled with MM6.15 and UIC2 (r=0.924, p=4.8x10^{-5}). Similarly, a very strong correlation was found between the proportion of MM6.15-positive and that of UIC2-positive cells in the samples (r=0.992, p=2.13x10^{-9}).

Functional study of Pgp pump

The function of Pgp pump in the cells derived from the ascites of the patients and that of MDR^{+} and MDR^{-} cell lines were determined by R123 substrate uptake of the cells in the presence and absence of the Pgp pump blocker CSA. For positive

control of Pgp expression and function NIH 3T3 MDR1 G185, while for negative control NIH 3T3, JY and HPBL cells were used. The mean fluorescence intensities of the CSA treated samples were normalized to the fluorescence intensities of the CSA untreated samples to generate a ratio, R_{R123}. CSA treatment increased the R123 uptake of the MDR$^+$ cells, while it did not modify the uptake of the MDR$^-$ cells. If the R_{R123} value is around 1 the function of the pump is negligible, if the value is remarkably higher than 1, it indicates intensive function of the pump.

The ratio of R123 of CSA treated and untreated samples were 8.1±3 in the case of NIH 3T3 MDR1 G185 cells (n=3). The original NIH 3T3 showed a significantly lowered drug uptake ratio (1.1±0.05, n+3, p<0.0001). R_{UIC2} ratios were 75±22, 1,08±0.1, 1.1±0.05, 1.02±0.1 for the NIH MDR1 G185, NIH 3T3, JY, and HPBL cells, respectively (n=3). The drug uptake of control human peripheral lymphocytes and JY cells, which do not express the Pgp pump, did not change significantly in the presence and absence of CSA.

We demonstrated the expression of Pgp in the membrane of cells derived from ascites using single cell fluorescence digital imaging. While single cell imaging is time consuming, flow cytometry based on extracellularly bound fluorescent markers provides reliable results supported by good statistics in short period of time. Therefore, although single cell imaging is also a possible method to detect Pgp expression in cells from ascites, we recommend it only as a useful additional method in special cases.

All of our patients had been treated with chemotherapeutic drugs before the collection of ascites samples. Bakeland et al. (2000) reported that the presence of Pgp could be found in 47% of the patients prior to chemotherapy and they considered the presence of it as a negative diagnostic sign. They also report that a part of the patients became Pgp-positive due to chemotherapeutic treatment. They found statistically significant positive correlation between Pgp positivity and the presence of ascites (p=0.013).

We suggest that the quantitative and functional method to detect MDR1/P170 mediated multidrug resistance in cells derived from ascites of malignant ovarian cancer patients can provide useful information for the more efficient treatment of the disease by proper adjustment of the chemotherapy protocol.

4.2 Paclitaxel modifies the accumulation of tumor-diagnostic tracers in different ways in Pgp-positive and negative cancer cells

Determination of Pgp expression level and function

Pgp was detected by means of indirect immunofluorescence, using a monoclonal antibody (UIC2) recognizing the extracellular epitope of the protein. The R_{UIC2} values were 28±8, 1.1 ± 0.1 and 1.1±0.2 for the A2780AD, A2780 and JY cells, respectively (mean±S.D., $n = 3$). The R123 uptakes of the A2780AD Pgp$^+$ and A2780 Pgp$^-$ cells were determined in the presence or in the absence of the Pgp modulators CSA or VER. The R123 accumulation of the Pgp$^+$ cells was lower than that of the Pgp$^-$ cells since the pump molecules were activated in the presence of substrates, e.g. R123, decreasing its net influx. When the Pgp pump was blocked by modulators (e.g. CSA or VER), the R123 uptake in the cells increased, whereas the R-123 accumulation of the Pgp$^-$ cells did not change significantly. Similar effects were observed when we used DNR, another substrate of the Pgp pump.

Effects of paclitaxel treatment on the accumulation of tumor-diagnostic radiotracers in cancer cells

We measured the kinetics of the ^{18}FDG uptake of the A2780AD and A2780 cell lines in the presence and in the absence of paclitaxel. After incubation for 60 min the ^{18}FDG uptake of the A2780AD cells was 73% higher than that of the A2780 cells. Paclitaxel treatment further increased the ^{18}FDG accumulation both of the A2780AD and A2780 cell lines, but the accumulation rate was higher in the Pgp$^+$ cells. Paclitaxel increased the ^{18}FDG uptake of the JY human B lymphoid cells. Incubation of the JY cells in the presence of 25, 50 or 75 μM paclitaxel for 30 min enhanced the ^{18}FDG uptake to 140, 160 and 170%, respectively, as compared with the control.

The effects of paclitaxel on the accumulation kinetics of ^{99m}Tc-MIBI uptake were determined. Paclitaxel increased the ^{99m}Tc-MIBI uptake of both of the Pgp$^+$ and the Pgp$^-$ cells in a concentration- and incubation time-dependent manner, but the increase was more pronounced for the Pgp$^+$ cells. Paclitaxel (1–70 μM) did not change significantly the ^{11}C-choline uptake of the three investigated cell lines during an incubation period of 30 min.

The results of the comparative study of the R123, DNR, ^{99m}Tc-MIBI, ^{18}FDG and ^{11}C-choline accumulation in the A2780AD and A2780 cells are summarized below. VER (50 μM) and CSA (20 μM) reinstated the accumulation of R-123 and DNR in the A2780AD Pgp$^+$ cells, but did not modify the accumulation of these agents in the A2780 Pgp$^−$ cells. CSA completely restored, whereas VER did not effect the accumulation of ^{99m}Tc-MIBI in the case of the Pgp$^+$ cells. Paclitaxel (70 μM) treatment reinstated the ^{99m}Tc-MIBI uptake of the Pgp$^+$ cells, and only mildly increased the uptake of ^{99m}Tc-MIBI in the Pgp$^−$ cells. Paclitaxel and CSA elevated the ^{18}FDG uptake of both cell lines, suggesting that this effect was not Pgp-dependent. In flow cytometric measurements, paclitaxel (25–75 μM) enhanced the R123 and DNR uptakes of the Pgp$^+$ cells to 200% and 150% of the control, respectively. Paclitaxel decreased the R-123 and DNR uptakes of the Pgp$^−$ cells to about 80%, suggesting that the increased accumulation of R123 and DNR in the Pgp$^+$ cells is Pgp dependent. Paclitaxel (10–70 μM) and CSA treatment did not modify the choline uptake of the Pgp$^+$ and Pgp$^−$ cells. VER treatment decreased the choline accumulation of the Pgp$^+$ and Pgp$^−$ cells, suggesting that the VER effect is Pgp independent. In our experiments, paclitaxel restored the ^{99m}Tc-MIBI accumulation of the Pgp$^+$ human ovarian cancer cells in a concentration-dependent manner and also increased the ^{99m}Tc-MIBI uptake of the Pgp$^−$ cells, though to only a moderate extent. The slight increase of the ^{99m}Tc-MIBI accumulation in the Pgp$^−$ cells may be explained by aspecific effects of paclitaxel on the plasma membrane structure or permeability.

Pgp is a transporter molecule responsible for paclitaxel extrusion from tumor cells, but several other mechanisms may be responsible for the clinically observed paclitaxel resistance, including molecular changes in β-tubulin, variations in apoptotic regulatory and mitosis checkpoint proteins, etc. Our results suggest that paclitaxel is a high-affinity substrate for Pgp, since it is a good competitor of Pgp-mediated ^{99m}Tc-MIBI extrusion from Pgp$^+$ cells. Paclitaxel also increased the uptakes of R123 and DNR in a Pgp-dependent manner, but to a lesser extent as compared with ^{99m}Tc-MIBI. The various extents of the effects of paclitaxel on the accumulation of different Pgp substrates may be explained by supposing more than one substrate binding site (three or four), probably forming a large complex drug-binding pocket.

Taken together, our results indicate that paclitaxel modifies the uptakes of tumor-diagnostic tracers (^{18}FDG and ^{99m}Tc-MIBI) in both Pgp-dependent and independent manners, whereas it does not modify the uptake of ^{11}C-choline. An interpretation of the effects of paclitaxel can promote the correct in vivo diagnosis of Pgp$^+$ and Pgp$^-$ tumors.

4.3 Comparative analyses of kinetic models to study glucose metabolism of the brain

A unique feature of PET is the possibility for the in vivo quantification of tissue radioactivity converting these data into quantitized parameters of biochemical or physiological processes. There are alternative kinetic models available. In our experiments we compared three kinetic models to calculate the brain metabolic activity on five volunteers. The models were the following: the most general model the so called Phelps analysis, the rapid calculation the so called Patlak method and the Standard Uptake Value calculation the so called SUV method.

Using correlation analyses we proved that the rapid Patlak or SUV methods can provide appropriate assumption of the data generated by the Phelps method in certain regions of the brain. Both of the simplified methods gave distortion (compared to the Phelps method) at the same anatomical regions of the brain. These regions were the low metabolic activity regions of the white matter, regions situated close to a large blood vessel, or around large blood vessels in the basis of the brain [gyrus rectus (l.u.), pons, capsula interna (l.u.), cerebellum (l.u.), corpus callosum]. The possible reason of the distortion is that the simplified kinetic models neglect the degree of dephosphorilation of FDG-6P. They do not count with the presence of the amount of radiopharmacon in the tissue which appears as an incremental response due to the presence of the radioactivity of the vascular compartment. We found very low correlation values between the data gained by the Phelps method and the other two simplified methods in these regions. Our observations provide information for the clinicians regarding the regions of the brain in which the rapid simplified methods may be used for the assumption of the metabolic activity and in which regions should they be avoided. In the latter cases it is advised to stick to the time consuming but very precise Phelps method. These results may contribute to the elaboration of a

procedure which measures the capacity of the Pgp pump in tumors through the FDG uptake.

6. Summary

In our experiments we detected the presence and measured the function of the P-glycoprotein using fluorescence and isotope techniques.

We showed for the first time in clinical samples that the Pgp is present to a different extent in the ascites cells of patients with ovarian cancer. The percentage of Pgp positive cells in the ascites of patients treated with chemotherapy was between 10-79%. The expression of the Pgp pump was proved by indirect immunofluorescent method while the function of the pump by the detection of the accumulation of R123 fluorescence dye in the cells. We found strong correlation between the rate of expression and function of the protein.

We observed higher ^{18}FDG PET radiotracer accumulation in the Pgp$^+$ human adenocarcinoma-derived ovarian cell line A2780AD than in its Pgp$^-$ counterpart A2780. Paclitaxel treatment further enhanced the difference in ^{18}FDG accumulation but did not influence the accumulation of the ^{11}C-choline tumor-diagnostic PET radiotracer. The accumulation of the ^{99m}Tc-MIBI Pgp substrate SPECT radiotracer was significantly lower in the A2780AD Pgp$^+$ positive cells than in their Pgp$^-$ counterparts. Paclitaxel treatment influenced the accumulation of ^{99m}Tc-MIBI through Pgp dependent and Pgp independent ways. Based on our observations we may draw the conclusion that paclitaxel treatment influences the accumulation of the tumor-diagnostic tracers in different ways in Pgp positive and negative cells which should be taken into consideration upon coming to correct diagnostic decision.

We carried out detailed analyses of the same set of data using three different methods to quantitize the FDG accumulation in different regions of the human brain. Using correlation analysis we proved that the simplifying methods such as the SUV and Patlak methods can only be used in certain regions of the brain with good correlation to the general Phelps method. We also proved that both of the simplifying methods lead to distortion in the same regions of the brain. The possible reason of the distortion is that the simplifying methods do not take into consideration the degree of dephosphorilation of FDG-6P which can be relatively high in the vicinity of vascular compartments.

7. Publications

The thesis was based on the following publications

Krasznai ZT, Péli-Szabó J, Németh E, Balkay L, Szabó G, Goda K, Galuska L, Trón T, Major T, Hernádi Z. Paclitaxel modifies the accumulation of tumor-diagnostic tracers in different ways in P-glycoprotein-positive and negative cancer cells . Eur. J. Pharm. Sci. 28, 249-256, 2006.

Impact factor: 2.341 (2005)

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Márián T, Balkay L, Szabó G, **Krasznai ZT,** Hernádi Z, Galuska L, Szabó-Péli J, Ésik O, Trón L, Krasznai Z. Biphasic accumulation kinetics of [^{99m}Tc]-hexakis-2-methoxyisobutyl isonitrile in tumour cells and its modulation by lipophilic P-glycoprotein ligands. Eur. J. Pharm. Sci. 15, 201-209, 2005.

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Cumulated impact factor of the publications: **22. 697** (JCR, 2005).

Number of citations: **18**