Immunosassay of P-glycoprotein on single cell by capillary electrophoresis with laser induced fluorescence detection

Hua Xiao a, Xin Li a, Hanfa Zou a,∗, Ling Yang b, Yanqin Yang b, Yulin Wang b, Hailin Wang c, X. Chris Le c

a National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
b Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
c Department of Public Health Sciences, Faculty of Medicine, University of Alberta, 10-102 CSB, Edmonton, Alberta T6G 2G3, Canada

Received 25 July 2005; received in revised form 15 September 2005; accepted 18 September 2005
Available online 21 October 2005

Abstract

The cellular mechanism based on P-glycoprotein (PGP) for its drug pump function has become very important in multidrug resistance (MDR) research. A method has been established to characterize PGP on single K562 cell by coupling capillary electrophoresis with laser induced fluorescence detection. A permeable intact cell after the immunoassay binding with fluorescence labeling antibody was injected into the capillary and directly separated without lysis. It was found that once 5–10 optional cells were detected in batch, the PGP amount on this cell line could be outlined and calculated clearly. The PGP amount on K562 MDR cell line is 3.88 times higher than that on K562 sensitive cell line. These two cell lines with immunoassay binding were also analyzed by injection of multi-cells in order to improve the throughput. A resistance factor so called multidrug resistance multiple (MRM) was introduced to evaluate the MDR difference between cell lines. The MRM values of the cell line K562 measured by single cell analysis are well correlated with those by flow cytometry, which also prove the validity of our method in single cell analysis for the possibility of cancer diagnosis, pharmacokinetics and drug screening in future.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Capillary electrophoresis; Laser induced fluorescence detection; Multidrug resistance; P-glycoprotein; Resistance factor

1. Introduction

Multidrug resistance (MDR) is defined as resistance of tumor cells to the cytostatic or cytotoxic actions of multiple, structurally dissimilar and functionally divergent drugs commonly used in cancer chemotherapy [1]. For some malignancies, of which colon cancer is a notable example, the drug resistance appears to be an inherent property of the tumor cells. MDR is the most important and challenging topic in cancer treatment research today [2]. Most cancer deaths result from the cancer’s either being intrinsically resistant to chemotherapeutic drugs or becoming resistant after being initially sensitive [3]. MDR is termed “intrinsic” when the disease is refractory to chemotherapy from the outset, or “acquired” when the disease becomes insensitive to treatment upon relapse. MDR is responsible for the overall poor efficacy of cancer chemotherapy. Attempts to develop drugs targeting proteins that mediate MDR were initiated as soon as such proteins began to be identified [4].

With dependence on the sites of function, there are several cellular mechanisms to explain how the MDR work: (1) enhanced efflux of drugs by the human multidrug-resistance-associated protein (MRP), P-glycoprotein (PGP) is a good example [5]; (2) alterations in drug targets, such as DNA topoisomerase II [6]; (3) increased detoxification of compounds, e.g. by the glutathione system and overexpression of the human vault protein LRP [7]; (4) enforced ability to repair damnification of DNA [8]; (5) the generation of anti-apoptosis mechanism [9].

The classic form of MDR is related to the overexpression of the MDR-1 gene, which encodes for a 170 kDa glycoprotein termed PGP for its drug pump function in MDR. PGP is responsible for transporting a variety of amphiphilic substances across plasma membranes [10] and functions as a transmembrane drug efflux pump. PGP has 12 transmembrane domains contained in two homologous halves and two ATP-binding cassette domains.
Its primary sequence and structure are highly conserved to members of the superfamily of ATP-binding cassette transmembrane transporters [11]. There have been some methods to characterize the PGP on cell membrane, such as flow cytometry, general fluorescence method, etc [12–14].

Flow cytometry can characterize PGP on cells very quickly. But the obtained results are the average value of the 100,000 cells. Generally, microbeads were selected as model by flow cytometry to simulate cell surface for PGP quantitation. General fluorescence method needs lys of the cells before determination that will lead invalidity. Western blotting can only semi-quantitate the PGP on cells by chemiluminescence. Most of them cannot quantitate PGP on single cell level. At the same time, there are seldom reports on PGP characterization by separation methods still now.

The use of separation methods to characterize the composition of a single cell has a 50-year history [15–17]. Since Jörgenson and co-workers [18] first used capillary electrophoresis (CE) for the analysis of a single neuron, it has become an important tool in studying cellular chemistry [19,20]. There are two approaches in such studies: bulk analysis of a large number of cells using cellular extracts [21–23] and single cell analysis [18,24,25]. CE with laser induced fluorescence (LIF) detection system has become very predominant in single cell analysis [26–28]. It is available to use its separation ability to characterize the tiny metabolism changes in cells [27]. DNA, RNA, proteins, amines, amino acids and steroids also have been detected by this method [19,21,29,30].

In this paper, we report a method to characterize the PGP on K562 cell line by coupling CE with LIF detection. With the analysis of more than 5–10 single cells, the K562 MDR cell line (K562/A) and K562 sensitive cell line (K562/S) can be well distinguished and characterized. The most predominant advantage of our method is that the total amount of PGP on single cell can be detected and calculated. This is helpful for us to understand the cancer drug pump mechanism and establish a choice of models for drug screening on single cell level.

2. Experimental section

2.1. Apparatus

The CE system is consisted of a 0–30kV power supply (GYW-030, CAS Power Supply Factory, Beijing, China), a laser fluorescence detector from Picometrics (ZETALIF, Ramon Ville, France) and laser source of an Ar-ion 488 nm beam from Spectra-Physics (Mountain View, CA, USA) operated at 6mW. The two electrodes were fixed on one plane. One electrode was fixed above an inverted microscope (Olympus, Tokyo, Japan) slide. Capillary was controlled by a micromanipulator (MN-151, Narishige, Setagaya-ku, Japan). A two-way valve (HV3-2, Hamilton Company, Reno, NV, USA) connected the outlet of the capillary with the outlet buffer vial and a 106 cm water column to generate siphoning force. Under the siphoning force, a single cell can be injected into the capillary with the observation by inverted microscope.

Fused-silica capillaries with 365 μm (o.d.) and 25 or 75 μm (i.d.) with effective length 50 cm (65 cm total length) (Yongnian Optic Fiber Plant, Hebei, China) were used in this work. The capillary was first treated with 0.2 M sodium hydroxide for 1 h, and washed with methanol then water for 1 h, respectively. Later the activated capillary was reacted with γ-methacryloxypropyltrimethoxysilican according to the procedures described by Hjerten [31]. After reaction at 45 °C overnight, the capillary was washed with 500 μl methanol and then filled with a 4% acrylamide solution containing 0.1% ammonium persulphate and 0.1% N,N,N′,N′-tetramethylethylenediamine. After polymerization overnight, the capillary was rinsed with water and excessive polyacrylamide was pushed out of capillary.

2.2. Reagents

Unless otherwise stated, all chemicals were analytical grade. Mouse raised JSB-1 antibody (Cat. Nr. MON 9011) was purchased from Beijing Zhongshan Company (Beijing, China). The goat anti-mouse IgG-FITC (GAMIF) was purchased from Sino-American Biotechnology Co. (IF9030, Beijing, China). They were kept at −20 °C for storage. The antibody JSB-1 bind with a conserved cytoplasmatic epitope (which is located on the inner surface of cell membrane) of the plasma membrane-associated 170–180 kD glycoprotein, the expression of which is strongly correlated with the degree of cell line of MDR. Five microlitres of JSB-1 and 5 μl of GAMIF solution were diluted to 500 μl with PBSBN, respectively, which contains 5% BSA and 0.1% N,N,N′,N′-tetramethylethylenediamine. After microfiltration overnight, the capillary was rinsed with water and excessive polyacrylamide was pushed out of capillary.

2.3. Cell culture

The K562 cell line was bought from the Institute of Blood, Chinese Academy of Medical Sciences (Tianjing, China). K562/A was cultured with RPMI-1640, supplemented with 10% fetal calf serum and domesticated with 5 μg/ml adriamycin at 37 °C in 5% CO2 atmosphere. The culture of K562/S was followed K562/A with the exception of domestication without 5 μg/ml adriamycin. The cells were washed three times with PBS to remove the residue from the medium before the experiments.

2.4. PGP fluorescence immunosay

The K562/A and K562/S were grown to 80% confluence and harvest in 1 ml tube, about 5 × 105 cells each. After washed with PBS for three times, the supernatant was removed. A 90 μl of PBS and 10 μl FACS permeabilizing solution were added for incubation of 10 min to make the cell membrane more perme-
able. The cell solution was centrifuged at 2500 rpm for 3 min and
the supernatant was removed. Then 100 μl of the diluted solution
JSB-1 was added and incubated at 37 °C for 1 h. It was washed
with PBSBN three times to remove the JSB-1 non-specific
adsorption. Cell solution was centrifuged and the supernatant
was removed again. Then 100 μl of diluted GAMIF solution
was added. Cell solution was incubated at 37 °C for another
1 h. Finally, cell solution was washed with 500 μl PBSBN three
times to remove the GAMIF non-specific adsorption. The cells
were stored at 4 °C and ready for analysis. K562/A and K562/S
were treated according to the procedures stated above. Control
groups of K562/A and K562/S cells (cK562/A and cK562/S,
respectively) were also treated with the same procedures with
the exception of without incubation with JSB-1 antibody solu-
tion.

2.5. Single cell experiment

For single cell experiments, the cell suspension was diluted
with PBS to a final density about 10^5 cells/ml. Single cell injec-
tion was performed by a micromanipulator within the field range
of an inverted microscope. First, we modulate the capillary inlet
just above a proper cell chosen. Then move down the capillary
tip to the cell in about 10 μm distance. A single cell was injected
into the capillary with a 10.8 kPa × 1 s siphoning pulse created
by the 106 cm water column, which was connected with the out-
et of the capillary and the outlet buffer vial through the two-way
valve. Turning the valve on, a cell was drawn into the capillary
tip for CE-LIF analysis at the applied voltage of 100 V/cm.

3. Results and discussion

3.1. Antibodies and immunoassay

Mixed solutions with different ratios of GAMIF and JSB-1
were prepared and left at room temperature for incubation in
dark overnight. Fig. 1 presents the capillary electropherograms
of GAMIF and its conjugate with JSB-1. By comparing the
three electropherograms, it can be found that with the increasing
amount of JSB-1 in the mixed solutions, the peak height of their
conjugate increased. When the ratio of JSB-1 to GAMIF is 1:10,
there is almost no conjugate (peak 2) observed. But when their
concentration ratio is 1:2, GAMIF has almost completely bound
with JSB-1, and there is almost no addition (peak 2) observed. By comparing the
detection for the conjugate of GAMIF with JSB-1 is 40 zmol.

Fig. 1. Electropherogram for analysis of GAMIF and its conjugate with JSB-
1 by CE-LIF. Capillary, 65 cm × 75 μm (i.d); separation voltage, 150 V/cm;
injection, 30 V/cm for 5 s; separation buffer, 0.01 M Tris-0.01 M boric
acid (pH 7.4). GAMIF was incubated with JSB-1 for immunobinding for
overnight. Peaks: (1) GAMIF; (2) conjugate of GAMIF with JSB-1. Samples:
(A) JSB-1:GAMIF = 1:10 (up); (B) JSB-1:GAMIF = 1:2 (middle); (C) JSB-
1:GAMIF = 1:1 (down) by keeping GAMIF concentration at 1.5 μM.

GAMIF and its conjugate with JSB-1 were used to evaluate
the reproducibility of our system, the relative standard deviations
(R.S.D.) of the migration time for GAMIF and its conjugate with
JSB-1 were 2.43 and 5.46% with consecutive five injections,
respectively, and the R.S.D. of peak areas were 2.46 and 5.38%.

3.2. Detection limits and reproducibility

Based on the electropherograms for separation and detection
of low nanomolar concentration of antibodies, the concentra-
tion detection limits for antibodies were determined within the
range of 10^{-10}-10^{-11} M with a S/N of 3, and the mass limit of
detection for the conjugate of GAMIF with JSB-1 is 40 zmol.

3.3. The incubation condition of immunoassay

The influence of FACS™ permeabilizing solution on PGP
immunoassay has been investigated. Because the antibody JSB-
1 only bind with a conserved cytoplasmic epitope of the plasma
membrane-associated 170–180 kD glycoprotein, it is necessary
to make the cell membrane more permeable for the immunobind-
ing between JSB-1 and the PGP antigen. FACS™ permeabi-
liter solution was used to improve the permeability of cell
membrane. Two groups of K562/A and K562/S cells were used
to evaluate the influence of FACS™ permeabilizing solution on
the reproducibility of our system, the relative standard deviations
(R.S.D.) of the migration time for GAMIF and its conjugate with
JSB-1 were 2.43 and 5.46% with consecutive five injections,
respectively, and the R.S.D. of peak areas were 2.46 and 5.38%.

3.3. The incubation condition of immunoassay

The influence of FACS™ permeabilizing solution on PGP
immunoassay has been investigated. Because the antibody JSB-
1 only bind with a conserved cytoplasmic epitope of the plasma
membrane-associated 170–180 kD glycoprotein, it is necessary
to make the cell membrane more permeable for the immunobind-
ing between JSB-1 and the PGP antigen. FACS™ permeabi-
liter solution was used to improve the permeability of cell
membrane. Two groups of K562/A and K562/S cells were used
to evaluate the influence of FACS™ permeabilizing solution on
cell membrane permeability. Before PGP immunoassay, the group of sam-
ple cells was suspended in 1/10 time of FACS™ permeabilizing solution and kept in dark for 10 min at room temperature. The
cell group of cells does not have the permeabilizing proce-
dure.

The amount of PGP on the two groups was determined by
CE-LIF and the obtained results were shown in Fig. 2. It is
indicated that the immunobinding of PGP on the permeabilized
cells is 5.55 times higher than that of unpermeabilized cells,
which indicates that the permeability of cell membrane is one of
the most important factors to affect the immunoassay of PGP
on cells.

The effect of incubation time of antibodies to bind with PGP
on cells was also investigated. It was found that incubation time
has a dramatic effect on the experimental results. Statistical pat-
terns were obtained with different incubation time as shown in
3.4. Single cell analysis

TB was used as running buffer in the experiments. The buffer can keep the permeabilized cells intact in the whole procedure. Thus the fluorescence of one cell can be detected after injection, including specific and non-specific binding.

At first, we injected one cell into the capillary tip by 10.8 kPa $\times$ 1 s siphoning. Then the intact cell moved toward the cathode under electric field, thus the electropherograms of the different cells could be obtained. It is something like flow cytometry, except the cell moves under high voltage. The typical electropherograms for single cell analysis were shown in Fig. 4.

Under the electrophoresis condition, one cell just presents one peak with very high peak height and very narrow peak width. In Fig. 4(A) and (B), the peak area of one cell is resulted from the total amount specific and non-specific binding of GAMIF and JSB-1. By subtracting the average peak area of the control cell group, the difference contributed by the specific and non-specific binding of JSB-1 with cell can be obtained. But the non-specific binding of JSB-1 usually makes very minor contribution to the difference, because JSB-1 is monoclonal antibody. Thus the difference can generally be considered as the total amount of PGP on single K562 cell all the time.

The statistical pattern for distribution of peak area contributed by PGP on single cell was described in Fig. 5. On the whole, the peak area on every cell line is distributed on a relatively centralized area. If enough cells were analyzed, a confidence interval may be established for each cell line. This also demonstrates specificity of our method due to the specificity of the first antibody JSB-1 and the use of fluorescent GAMIF.

3.5. Relative standard derivation of single cell analysis

Cell to cell difference is absolutely a big problem in single cell analysis. Cell has its intrinsic individuality so that it is inevitable to be different from each other. Even under the same domestication conditions, one cell may over express PGP while another one express little. Relative standard derivation of PGP for K562/A cell line is ranged from 20 to 122%, and that is 24–87% for K562/S.
Fig. 4. Electropherograms for analysis of single K562/A and K562/S cell using TB as separation buffer. Experimental conditions are the same as in Fig. 2.

3.6. PGP amount and resistance factor

Flow cytometry is a technology that simultaneously measures multiple characteristics of single cells rapidly. It has been widely used in cell biology, especially in fluorescence immunosassay of DNA, RNA, protein, etc. There have been a number of reports by flow cytometry to study MDR[12,32]. Flow cytometry was also adopted to measure PGP on cells in our case, and the obtained results were compared with those from single cell analysis. The value obtained from flow cytometry is not an absolute value of PGP on cells, but the average fluorescence intensity value of more than 10,000 cells. The value obtained from CE–LIF is a concrete amount of PGP.

The difference between K562/A cells and cK562/A cells ($A$) is the total amount of PGP on K562/A. The difference between K562/S cells and cK562/S ($S$) is the total amount of PGP on K562/S. It could be found that ($A$) is several times higher than ($S$), which implied that there were more MDR-1 gene and more expressed PGP on K562/A cells than K562/S cells. Based on the electrophoresis of JSB-1 with GAMIF, the molecular numbers of PGP on single cell can be calculated by the immunobinding at the stoichiometry of 1:1. The determined amounts of PGP are about $9.49 \times 10^{-18}$ mol (157,000 molecules) on single K562/A cell and $2.85 \times 10^{-18}$ mol (47,000 molecules) on single K562/S cell. Since the total amount of PGP on single cell could be obtained by the present method, some kinetic experiments, such as drug adsorption and drug metabolism can be carried out quantitatively. This also provides us a new parameter to estimate the process of ADME (adsorption, distribution, metabolism and excretion) and pharmacokinetics. The most important is that it can promote the MDR mechanism research, even on single cell level.

If K562/S cell line do not express PGP and JSB-1 do not have non-specific binding at the same time, then ($S$) = 0. But in fact after a period of domestication, K562/S can also express PGP to a certain degree. JSB-1 also has few non-specific binding. Then multidrug resistance multiple (MRM) was introduced as resistance factor to evaluate the MDR degree of cells lines, which was expressed as follows [33]:

\[
\text{MRM} = \frac{A - A_c}{S - S_c}
\]

The value of MRM can be regarded as the MDR comparison between different cell lines. It was calculated by experimental data that the amount of PGP on K562/A cells is 3.88 times higher than that on K562/S cells, which implied that PGP is over expressed on K562/A cells. MRM can be also applied for evaluation of domestication efficiency for different cell lines.
MRM is a good criterion to differentiate the MDR between cells. Relationship of MRM values obtained by single cell analysis with CE–LIF and multi-cell analysis by flow cytometry are shown in Fig. 6, and they are well correlated with each other.

### 3.7 Multi-cell electrophoresis

Although cell analysis one by one can detect PGP amount on single cells, the throughput is quite low. Under the force of electrical field, cells will move in turn according to their charge, size, shape, etc. When multi-cells were injected, the separation will be carried out based on electrophoretic mechanism. The throughput of analysis can then be improved by multi-cell electrophoresis.

In our multi-cell electrophoresis experiment, 12 cells were injected into capillary for cell separation. Those 12 cells were injected one by one at one time with some distance gap by controlling the capillary inlet and the siphoning force. The obtained electropherograms of K562/A, K562/S and their control group were shown in Fig. 7. Accordingly, the MRM value was calculated from the data of multi-cell electrophoresis as 3.25; this value is slightly lower than 3.88 by single cell analysis, but they are still comparable. This indicates that multi-cell electrophoresis can also be used to detect MRM of different cell lines. Multi-cell electrophoresis in fact is a kind of single cell analysis, which also analyzes cells on single cell level.

When single cell analysis and multi-cell electrophoresis were compared, the obvious difference in cell average peak areas was found. The average peak area of cells determined by single cell analysis is larger than that by multi-cell electrophoresis. This may result from cells binding during multi-cells injection and separation, which will result in relative smaller peak area of cell binding. The cell binding during cell electrophoresis can be supported by the phenomenon that the number of electrophoresis
peaks is sometimes smaller than the number of injected cells. Even though, the MRM values measured by CE–LIF with injection of single cell and multiple cells are quite close, which further means that the MRM values measured with single cell analysis, multi-cell electrophoresis and flow cytometry are well correlated each other.

4. Conclusions

Immunoassay for detection of PGP on single K562 cell was established by using CE–LIF. So-called MRM was introduced to evaluate the MDR degree of cell lines. This parameter is helpful in ADME research. The MRM values measured with single cell analysis, multi-cell electrophoresis and flow cytometry are well correlated each other. Compared with the method for fluorescence measurement of PGP on K562 cells by flow cytometry, the total amount of PGP on single cell can be well detected and calculated by CE–LIF. Based on the combination of principle of immunoassay with CE–LIF, many other cell lines can be analyzed and characterized on single cell level. Thus the developed method may be useful in the research of multidrug resistance mechanisms.

Acknowledgments

The financial supports from the National Natural Sciences Foundation of China (No. 205210120203), the China State Key Basic Research Program Grants (2003CB716002), and the Knowledge Innovation Program of DICP to H.Z. are gratefully acknowledged.

References