RESEARCH NOTES

Phospholipid flippase associates with cisplatin resistance in plasma membrane of lung adenocarcinoma A_{549} cells*

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Abstract The fusion of the liposomes containing (N-(7-nitro-2, 1, 3-benzoxadiazol-4-yl)-1,2-hexadecanoyl-Sn-glycero-3-labeled phosphatidylethanolamine (NBD-PE) with A_{549} and A_{549}/DDP cells was performed, and the activity of the phospholipid flippase in the plasma membrane of the cells was measured by fluorescence intensity change of NBD-PE in the outer membrane. When A_{549} or A_{549}/DDP cells containing NBD-PE were incubated at 37℃ for 0, 30, 60 and 90 min, the fluorescence intensities in the outer membrane of the cells were 0%, 1.4%, 2.9% and 7.8% for A_{549} cells, and 0%, 10.5%, 15.5% and 18.3% for A_{549}/DDP cells respectively, demonstrating that the phospholipid flippase was distributed in the plasma membrane of A_{549} cells, but its activity in the drug-resistant A_{549}/DDP cells was much higher than that in the A_{549} cells. When the A_{549}/DDP cells were incubated with a multidrug resistance reverse agent, verapamil, for 60 min at 37℃, the results showed that the NBD-PE in outer membrane decreased by 25.0% compared with the control's. Furthermore, when A_{549}/DDP cells were incubated with 25 μmol/L cisplatin, which is a specific anticancer drug, the flippase activity decreased by 31.6%, and it further decreased with the increase of cisplatin concentration, suggesting that phospholipid flippase in the membrane might be related to the cisplatin-resistance of human lung adenocarcinoma cancer cells.

Keywords: cisplatin-resistance, phospholipid flippase, human lung adenocarcinoma A_{549} cells.

During the chemotherapy treatment of cancer, the tumor cells are resistant not only to the used therapeutic drug but also to multiple apparently unrelated drugs with no obvious structural resemblance. The multidrug resistance (MDR) of tumor cells remains an serious cause of treatment failure in clinical cancer chemotherapy. The development of MDR is commonly associated with the high expression of a kind of ATP-binding cassette (ABC) proteins which can "pump" the intracellular anticancer drugs out of cells thus reducing the drug toxicity[1, 2]. MDR gene has been identified in the mammalian drug-resistant cell lines in addition to the overexpression of MDR transporting protein in the plasma membrane[3]. The mechanism of cancer MDR is still unclear up to now[4].

Higgins and Gottesman[5] first raised a hypothesis that a multidrug transporter is a flippase, which was demonstrated in different cell lines. In this study, by fusing liposomes containing fluorescence labeled phospholipid molecule NBD-PE with the plasma membrane of human lung adenocarcinoma cancer cells, we measured the activity of phospholipid flippase in the plasma membrane of A_{549}

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and A_{549}/DDP cells, and its relation with the cisplatin-resistance of the cells was also studied.

1 Materials and methods

1.1 Reagents and cell lines

Asolectin and cisplatin were from Sigma Co. NBD-PE (N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-hexadecanoyl-Sn-glycero-3-phosphatidylethanolamine) was purchased from Avanti Polar-lipids Co. The other reagents were of analytical grade. The human lung adenocarcinoma A_{549} or A_{549}/DDP cells were from Beijing Institute of Cancer Research. The resistant A_{549}/DDP cells were derived from wild type sensitive A_{549} cells using a stepwise selection protocol of increasing cisplatin concentration[6].

1.2 Preparation of liposomes containing NBD-PE and its fusion with the cells

Briefly, 800 μL of purified asolectin (125 mg/mL) dissolved in the chloroform:methanol (4:1) were mixed with 100 μL of NBD-PE (1 mg/mL). It was evaporated to dryness by nitrogen gas under vacuum. 3 mL buffer (130 mmol/L NaCl, 40 mmol/L Tris-HCl) were added into it, then sonicated until clear in ice-water bath to get the liposomes containing 3.5 nmol/L NBD-PE and 33 mg/mL asolectin. A 2 mL of A_{549} or A_{549}/DDP cell suspension (1×10^6 cells/mL) was respectively mixed and incubated with 200 μL of the NBD-PE-labeled liposomes for 30 min on ice, then centrifuged at 1000 *g* for 10 min. The pellets were rinsed twice with PBS buffer and used for the assay.

1.3 Assay of flippase activity

The flippase activity was determined by the methods of Ruetz and Suzuki et al[7, 8]. The fluorescence change of A_{549} and A_{549}/DDP cells labeled with NBD-PE was monitored using a Hitachi F-4010 fluorescence spectrophotometer equipped with a temperature-controlled water jacket and a magnetic stirrer. Fluorescence intensity was recorded at 530 nm (Ex) and 470 nm (Em) at 15℃. Fig. 1 shows the basic principle of the flippase activity assay. The fluorescence intensity of the NBD-PE labeled cell suspensions was recorded (F_T), then 40 μL of 1 mol/L water-soluble and membrane-impermeant anion sodium dithionite ($Na_2S_2O_4$) were added to quench the fluorescence acyl-nitro group of NBD-labeled phosphatidylethanolamine in the outer leaflet of the cellular membrane, and the fluorescence intensity change was recorded (F_D). Then NBD-PE fluorescence in the inner leaflet of the cellular membrane was quenched by $Na_2S_2O_4$ after the cells disrupted by the addition of 80 μL 0.25% (w/v) Triton X-100, and the fluorescence intensity change was recorded (F_0). The increase in fluorescence intensity of NBD-PE in the outer leaflet of the cell membrane was calculated by the following equation:

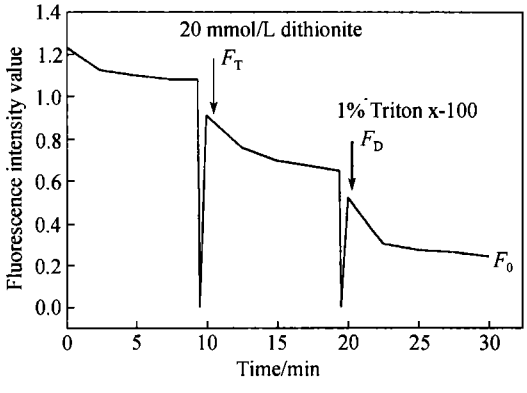

Fig. 1 Principle of assay for phospholipid flippase in plasma membrane.

Percentage (%) of NBD-PE in outer leaflet = $[(F_T - F_D)/(F_T - F_0)] \times 100$.

2 Results and disscussion

2.1 Increased activity of phospholipid flippase in A_{549}/DDP cells

At present, assay for the phosphatidylserine (PS) flip from inner to outer leaflet of the membrane is widely used to measure the phospholipid flip of cells undergoing apoptosis and aging[9]. To monitor the NBD-PE distribution change between the liposomes and human lung adenocarcinoma cancer cells, we used a method based on the fusion of liposomes containing NBD-PE with lung adenocarcinoma cancer cells by incubating them together. The flippase in the plasma membrane can transport the NBD-PE in inner leaflet of lipid bilayer to outer leaflet, so that the phospholipid flippase activity can be measured accurately based on the change of fluorescence intensity under normal or abnormal conditions. This is a more precise method than the PS flip assay used in recent years[10]. Fig. 2 shows the activity of phospholipid flippase of A_{549} and A_{549}/DDP cells incubated at 37℃ for 0, 30, 60 and 90 min. The results indicate a significant difference in phospholipid flippase activity in the plasma membrane between the two cell lines during the incubation period. The increase of NDB-PE in outer leaflet of the membrane in A_{549}/DDP cells is significantly higher than that in the A_{549} cells. The relative activity of phospholipid flippase in the plasma membrane of A_{549}/DDP cells is increased about 6 ~ 7 fold compared with that of A_{549} cells after 60 min incubation. Since Higgins and Gottesman et al. first proposed that multidrug transporter is a phospholipid flippase[5], more and more observations have been made in different laboratories. Ruetz et al. indicated that P-glycoprotein encoded by mouse MDR2 gene might be a phospholipid flippase, using NBD-PC (phosphatidylcholine) as an indicator[7, 11]. Helvoort et al. found that Pgp encoded by MDR1 gene was also a phospholipid flippase and P-glycoprotein encoded by the MDR3 gene was a phospholipid flippase specific for PC[12]. Recently, it was also reported that multidrug relative protein (MRP) as a drug transport protein can transport the NBD-labeled phospholipid to the outer bilayer of cells[13, 14]. The results here demonstrate that there exists a phospholipid flippase in the plasma membrane of human lung adenocarcinoma cancer A_{549} cells, and its significantly increased activity is associated with the drug-resistance of A_{549}/DDP cells.

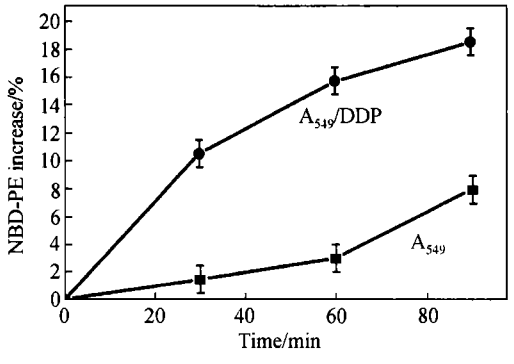

Fig. 2 Phospholipid flippase activity in plasma membrane of sensitive A_{549} cells and resistant A_{549}/DDP cells.

2.2 Increase of flippase activity in A_{549}/DDP cell membrane contributes to the cisplatin resistance

In order to further explore the relationship between the phospholipid flippase in the plasma membrane of the A_{549}/DDP cells and its cisplatin resistance, the translocation of NBD-PE was examined with or without verapamil and cisplatin. Verapamil, a calcium channel blocker, is widely used in basic medical and clinical researches of reversing multidrug resistance by competitively binding the substrate site of MDR transporter[15]. Verapamil and cisplatin can be "pumped" out of cancer cells as the substrate of MDR transporter. Therefore, both verapamil and cisplatin can decrease the translocation

of NBD-PE in the outer leaflet by competing the binding site with NBD-PE, which indicates that the phospholipid flippase is closely related to the MDR of cancer cells. As shown in Table 1, our results indicate that the translocation rate of NBE-PE for A_{549}/DDP cells treated with verapamil decreased by 25% compared with the control that was without the treatment of the substrates. The inhibition of the phospholipid flippase activity was more obvious with the increasing cisplatin concentration from IC20 (drug dose of 20% cell death efficiency) to IC50 (drug dose of 50% cell death efficiency). Our previous results indicated that the efflux rate of Rhodamine 123, a reagent used to assay the multidrug resistance of cancer chemotherapy, in the resistant A_{549}/DDP cells was faster than that in the sensitive A_{549} cells[16]. Our results suggest that the phospholipid flippase is closely associated with the transport activity of multidrug proteins in the plasma membrane of the A_{549}/DDP cells.

Table 1 Inhibition of phospholipid flippase activity in the plasma membrane of A_{549}/DDP cells with different substrates

	Control	Verapamil	Cisplatin(IC20)	Cisplatin(IC50)
Increase of NBD-PE/%	15.5 ± 2.6	11.6 ± 2.1	10.6 ± 1.9	3.2 ± 1.2
Inhibition/%	100	25.0 ± 2.2	31.6 ± 2.1	79.4 ± 3.2

Note: Data are expressed as the increase percentage of NBD-PE in the outer leaflet of the membrane of human lung adenocarcinoma A_{549}/DDP cells, 60 min at 37℃ with verapamil (20 μmol/L), cisplatin IC20 (25 μmol/L) and cisplatin IC50 (80 μmol/L). The values are the means ± SE ($n = 3$). $p < 0.05$ (verapamil), $p < 0.01$ (cisplatin).

Multidrug resistant mechanism is commonly associated with the multidrug transporter expressed in the plasma membrane which can actively pump drugs out of cells by an ATP-dependent process. In recent years, other multidrug resistance-related proteins like MRP[17], LRP[18] were found in addition to the 170 kD P-glycoprotein of the ATP-binding cassette (ABC) superfamily. But which proteins and how they function in the resistance to cisplatin in the human lung adenocarcinoma A_{549}/DDP cell line are not determined[18, 19]. This study provides evidence for the existence of a phospholipid flippase in the membrane of human lung adenocarcinoma A_{549} cells and shows that the change in its activity is responsible for A_{549}/DDP cell's resistance to cisplatin. The experimental data may provide new information and clue for further exploring the molecular mechanism of multidrug resistance of human lung adenocarcinoma A_{549} cells.

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