ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. LVII, 20 SECTIO D 2002

Department of Clinical Immunology, University School of Medicine, Lublin
Zakład Immunologii Klinicznej Akademii Medycznej w Lublinie

ROBERT PIEKARSKI, JACEK ROLIŃSKI, MICHAŁ DRAGAN, PIOTR POŻAROWSKI

Jc-1, the sensitive probe of p-glycoprotein function in hematological malignancies

Jc-1 — czuły wskaźnik funkcji glikoproteiny-p w chorobach rozrostowych układu krwiotwórczego

Multidrug resistance is the principal reason for treatment failure in patients with some hematological malignancies, particularly acute myeloid leukemia (AML), B-cell chronic lymphocytic leukemia (B-CLL), multiple myeloma, non Hodgkin's lymphoma and chronic myelogenous leukemia (CML) in blast crisis [4]. The complex phenomenon of MDR is related with the expression of various proteins. P-glycoprotein (P-gp), the product of the multidrug resistance-1 (MDR-1) gene has played a mayor role in the development of resistance mechanism [1]. P-gp is a member of the ATP-binding cassette (ABC) superfamily of transporter proteins, located in the plasma membrane, acts as an energy-dependent drug efflux pump [18]. Furthermore, other novel proteins may be associated with a MDR phenotype. One such protein, the multidrug resistance-associated protein (MRP-1), lowers intracellular drug accumulation by promoting drug efflux [15]. Recent studies have reported that MRP functions as a transporter of glutathione S-conjugates [23]. Another protein conferring MDR is the lung resistance protein (LRP). Recent studies have shown that LRP is the component of a major vault protein complex located in the cytoplasm and at the nuclear pore [15]. It has been hypothesized that LRP may confer resistance by altering drug transport between cytoplasm and the nucleus.

There are discrepancies between recent large clinical trials regarding the effect of MDR phenotype, measured by P-gp/MDR expression in the clinical course [18]. Some of these studies reported that P-gp expression is not a prognostic factor in AML [3]. Two studies found no association between P-gp and clinical drug resistance (Kato et al., Ito et al.). However, many studies have demonstrated no correlation between P-gp expression with poor response to therapy [17, 24, 25]. Another recent study concluded that P-gp/MDR has significant association with remission rates. Because of the

discrepancies among the results found, most researchers consider that methodology should be standardized and that all new studies should investigate at least two parameters: P-gp expression and P-gp functional assay. Recent studies have confirmed the prognostic value of both drug accumulation and P-gp expression (Guerci et al.). Jean Pierre Marie et al. maintained that the variations between studies may be explained partially by the use of different positive control cell lines and the number of units arbitrarily assigned to these controls [17]. Patient selection might also account for different results.

An analysis of P-gp expression in patients with ALL found low mean MDR_1 mRNA expression in primary ALL and first relapses of ALL and significantly higher expression levels in recurrent relapses of ALL [2]. No correlation between increased levels of MDR_1 mRNA and protein with clinical resistance has been observed in B-cell chronic lymphocytic leukemia (CLL) [19, 21]. Other studies have reported no association MDR phenotype with Rai stage, lymphocyte counts, duration of disease or disease progression [16, 21]. Webb et al. concluded that MDR_1/P-gp expression is involved in multidrug resistance phenomenon only after exposure to P-gp transportable drugs [22]. These findings suggest that P-glycoprotein overexpresssion in B-CLL is intristic rather than acquired.

P-gp protein acts as the efflux pump extruding range anticancer drugs from the cell against a concentration gradient (Juliano et al.). MDR phenomenon is referred to structurally and functionally unrelated cytotoxic drug such as anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vincristine, vinblastine), taxol, tamoxifen and actinomycin D. However, the researchers did not find cross-resistance to several other drugs such as chlorambucil, cyclophosphamide, antimetabolites (cytarabina, methotrexate) or cisplatin. A variety of compounds termed modulators, reversal agents or chemosensitizers are capable of reversing the drug efflux mechanism of P-gp protein. Such drugs include: tamoxifen, reserpine, verapamil, cyclosporine A and derivatives (PSC388), steroids (progesterone), vindoline and lipophilic cationic molecules like amiodaron or dypyridamole [2].

Many report discuss of the role of P-gp as a regulator of apoptosis [8, 20]. The authors have shown that P-gp expressing cells are resistant to cell death induced by various stimuli activated the caspase apoptotic cascade, but not resistant to caspase-independent cell death mediated by pore forming proteins and granzyme B [8, 20].

In *in vitro* systems, increased cytostatics concentration in the presence of modulators in MDR^+ tumour cells results in increased cell death. The problem is more complex in vivo, where tumour cells may develop additional resistance mechanisms affecting mechanisms regulating apoptosis. Then, often at advanced stages of the disease, increased intracellular concentration of the anticancer drug may not be sufficient to overcome resistance.

To evaluate P-gp function tests with anthracyclines, rhodamine 123 (Rh 123), 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$) or calcein-AM are used commonly [3, 13, 14]. These tests based on drug uptake are not sensitive enough for the detection of low-level resistant cells. In addition, resistant cells are distinguished from sensitive

ones only by comparison of mean fluorescence without any specific emission band [11]. In case of Rh 123, significant fraction of the dye binds to cell membranes, leading to high fluorescence background. Under these conditions they allow to distinguish two patient groups: sensitive and resistant one. Therefore, highly sensitive and specific P-gp functional assay, mainly in patients with low levels of markers for resistance, is still in demand.

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbocyanine iodide) — a cationic dye — exists as a monomer at low concentrations or at low membrane potential (green band of fluorescence). However, at higher concentrations (above 0.1 μM) or higher potentials, JC-1 forms red fluorescence centered at 590 nm. This carbocyanine dye initially was used to:

- Analyze of mitochondial potential changes especially in apoptosis,
- Detect human encephalomyophaty,
- Investigate mitochondrial poisoning, uncoupling and anoxia,
- Analyze the effects of drugs.

Kühnel et al. have shown that JC-1 is also a good probe of P-gp [11]. They have employed a specific property of JC-1: the dependence of its fluorescence emission wavelength on its concentration. The filtration of that dye prior to incubation with cells allows elimination of "J-aggregates" from the buffer. Then, all the red fluorescence detected results from "J-aggregates" formed within mitochondria (Fig. 1).

The functional test with JC-1 possesses unique for clinical diagnostics properties:

- Is easy to execute,
- Is not time-consuming,
- Does not require any washing step,
- Does not require additional efflux monitoring.

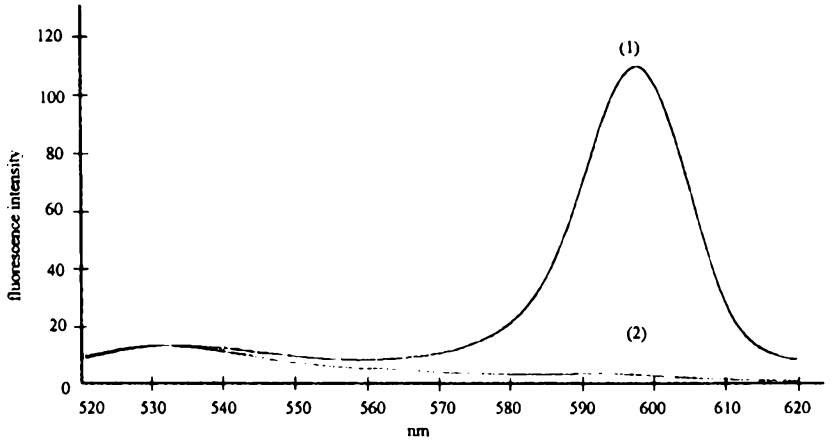

Fig. 1. Fluorescence emission spectra of JC-1 in phosphate-buffered saline before filtration (1) and after filtration on cellulose syringe filter (2)

Legrand et al. have shown that JC-1 may define 3 groups of patients: sensitive, resistant and intermediate-resistant [12]. Sensitive cells display both green and red fluorescence. In resistant cells, when P-gp activity increased, green band of JC-1 decreased and red was lost. In intermediate-resistant cells green fluorescence is identical to the sensitive cells, but red band was lost. The authors concluded that red fluorescence of JC-1 appeared to be more convenient for detection of low-level resistance and more sensitive than rhodamine 123 (Rh 123). This functional test allows comparison dye uptake in clinical samples in the absence and in the presence of modulators [11]. Such an assay could therefore be used for monitoring the effect of reversal agents.

In conclusion, novel functional assay of P-glycoprotein using JC-1 help us reach a better understanding of the role of P-gp.

REFERENCES

1. *Arceci R.J.*: Clinical significance of P-glycoprotein in multidrug resistance malignancies. Blood, 1993; 81, 9: 2215-2222.
2. *Beck J., Niethammer D., Gekeler V.*: MDR1, MRP, topoisomerase II alpha/beta and cyclin A gene expression in acute and chronic leukemias. Leukemia, 1996; 10 (suppl. 3): 39-45.
3. *Broxterman H.J., Sonneveld P., Feller N et al.*: Quality control of multidrug resistance assays in adult acute leukemia: correlation between assays for P-glycoprotein expression and activity. Blood, 1996; 87, 11: 4809-4816.
4. *Campos L., Guyotat D., Archimbaud E. et al.*: Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. Blood, 1992; 79, 2: 473-476.
5. *Damiani D., Michieli M., Michelutti A. et al.*: Expression of multidrug resistance gene (MDR1) in human normal leukocytes. Haematoogica, 1993; 78: 12-17.
6. *Guerci et al.*: Predictive value for treatment outcome in acute myeloid leukaemia of cellular daunorubicin accumulation and P-glycoprotein expression simultaneously determined by flow cytometry. Blood, 1995; 85: 2147-2153.
7. *Ito et al.*: Increased P-glycoprotein expression and multidrug-resistant gene (mdr1) amplification are infrequently found in fresh acute leukaemia cells. Sequential analysis of 15 cases at initial presentation and relapsed stage. Cancer, 1989; 63: 1534-1538.
8. *Johnstone R.W., Cretney E., Smyth M.J.*: P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. Blood, 1999; 93, 3: 1075-1085.
9. *Juliano R.L., Ling V.*: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochem. Biophys. Acta, 197; 455: 152-162.
10. *Kato et al.*: Absence of correlation between cytotoxicity and drug transport by P-glycoprotein in clinical leukemic cells. Eur. J. Haematol., 1991; 47: 146-151.
11. *Kuhnel J.M., Perrot J.Y., Faussat A.M. et al.*: Functional assay of multidrug resistant cells using JC-1, a carbocyanine fluorescent probe. Leukemia, 1997; 11: 1147-1155.
12. *Legrand O., Perrot J.Y., Simonin G. et al.*: JC-1: a very sensitive fluorescent probe to test Pgp activity in adult acute myeloid leukemia. Blood, 2001; 97, 2: 502-508.
13. *Legrand O., Simonin G., Beauchamp-Nicoud A. et al.*: Simultaneous activity of MRP1 and Pgp is correlated with in vitro resistance to Daunorubicin and with in vivo resistance in adult myeloid leukemia. Blood, 1999; 94: 31046-1056.
14. *Legrand O., Simonin G., Perrot J.Y. et al.*: Pgp and MDR activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. Blood, 1998; 91, 12: 4480-4488.

15. *Leith C.P., Kopecky K.J., Chen I.M. et al.*: Frequency and clinical significance of the expression of multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia. A Southwest Oncology Group Study. Blood, 1999; 94, 3: 1086-1099.
16. *Ludescher C., Hilbe W., Eisterer W. et al.*: Activity of P-glycoprotein in B-cell chronic lymphocytic leukemia determined by a flow cytometric assay. J. Natl. Cancer Inst., 1993; 85: 1751-1758.
17. *Marie J.P., Zittoun R., Sikic B.I.*: Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity. Blood, 1991; 78, 3: 586-592.
18. *McKenna S.L., Padua R.A.*: Multidrug resistance in leukaemia. Review. Br. J. Haematol. 1997, 96, 659-674.
19. *Michieli M., Raspadori D., Damiani D. et al.*: The expression of the multidrug resistance-associated glycoprotein in B-cell chronic lymphocytic leukaemia. Br. J. Haematol., 1991; 77: 460-465.
20. *Pallis M., Russell N.*: P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. Blood, 2000; 95, 9: 2897-2904.
21. *Sparrow R.L., Hall F.J., Siregar H. et al.*: Common expression of the multidrug resistance marker P-glycoprotein in B-cell chronic lymphocytic leukaemia and correlation with in vitro drug resistance. Leukemia Research, 1993; 17: 941-947.
22. *Webb M., Brun M., McNiven M. et al.*: MDR1 and MRP expression in chronic B-cell lymphoproliferrrrrative disorders. British Journal of Haematology 1998, 102, 710-717.
23. *Zhou D.C. et al.*: Expression of resistance genes in acute myeloid leukemias. Leuk. Lymphoma, 1994; 13 (suppl. 1): 27-30.
24. *Zhou D.C., Marie J.P., Subervillle A.M. et al.*: Relevance of mdr1 gene expression in acute myeloid leukemia and comparison of different diagnostic methods. Leukemia, 1992; 6: 879-885.
25. *Zhou D.C., Zittoun R., Marie J.P.*: Expression of multidrug resistance-associated protein (MRP) and multidrug resistance (MDR1) genes in acute myeloid leukemia. Leukemia, 1995; 9: 1661-1666.

STRESZCZENIE

Zjawisko oporności wielolekowej (MDR — *multidrug resistance*) polegające na oporności komórek nowotworowych na działanie różnorodnych chemioterapeutyków, pozostaje wciąż bardzo ważnym problemem i jest częstą przyczyną nieskutecznej chemioterapii. Problem oporności wiąże się głównie ze zwiększoną ekspresją glikoproteiny P (P-gp). Białko to należy do nadrodziny białek transportujących ABC (ATP — *binding cassette*), zlokalizowane jest w obrębie błony komórkowej i odpowiedzialne za usuwanie z wnętrza komórki wielu hydrofilnych związków, w tym chemioterapeutyków na zasadzie transportu aktywnego [1, 5].

Ludzkie Białko P-gp jest produktem genu MDR1, wchodzącego w skład nadrodziny genów MDR. Zwiększoną ekspresję P-gp oraz genu MDR1 stwierdza się w wielu nowotworach układu krwiotwórczego: ostrej białaczce szpikowej, przewlekłej białaczce limfatycznej B-komórkowej, przewlekłej białaczce szpikowej (zwłaszcza w okresie kryzy blastycznej), chłoniakach komórkowych [3].

Dużą rolę przywiązuje się do wczesnego wykrycia populacji komórek nowotworowych o fenotypie MDR w celu zastosowania właściwego leczenia. Dotychczas do badania funkcji P-gp używano testów z antracyklinami, rodaminą 123 czy kalceiną-AM [2, 8, 9]. Wyżej wymienione testy opierające się na wychwycie leków nie są wystarczająco czułe do detekcji komórek o niskim poziomie oporności, co stanowi podstawową wadę tych testów. Stosunkowo niedawno ski-

erowano szczególną uwagę na barwnik JC-1. Początkowo stosowany do oceny potencjału mitochondrialnego komórek, obecnie uważany jest za bardzo dobry wykładnik funkcji P-gp. Szczególną właściwością tego fluorochromu jest występowanie różnej długości światła emitowanego (zielonej lub czerwonej) w zależności od stężenia barwnika. Umożliwia to podział pacjentów na 3 grupy: lekoopornych, wrażliwych i o niskim poziomie oporności. Należy podkreślić, że istotny problem kliniczny stanowią pacjenci z niską ekspresją P-gp, u których może rozwinąć się fenotyp MDR. Stąd duże nadzieje pokłada się na teście funkcyjnym z JC-1, jako bardziej czułego i specyficznego w wykrywaniu przypadków o niskim poziomie oporności [7].