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In vitro detection of the MDR phenotype in rat myocardium: use of PCR, [^{3}H]daunomycin and MDR reversing agents

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A decrease in the intracellular drug concentration in resistant cells as compared to sensitive cells is one of the characteristics of the MDR phenotype. P-glycoprotein (Pgp) is thought to be responsible for an active efflux of some lipophilic drugs such as anthracyclines. Anthracyclines such as daunomycin are highly effective anticancer agents but induce a well-described, while incompletely explained, cardiac toxicity. In this study, we investigated the MDR phenotype in rat myocardium in terms of gene expression, detection of Pgp and indirect evaluation of Pgp function. A clear *mdr1a* gene specific expression in rat cultured myocardial cells and cardiac tissue was detected by RT-PCR. The incorporation of [^{3}H]daunomycin in myocardial cell cultures was studied with and without reversing agents. Daunomycin was found to have a high accumulation in cardiac cells illustrated by a C_i/C_e ratio of 2890. This high accumulation was moderately but significantly ($p < 0.05$) increased in the presence of a MDR reversing agent such as verapamil, PSC 833 or S9788. These results suggest that blockade of the Pgp in humans may result in an increased toxicity of several Pgp substrates in normal tissues like the myocardium.

Key words: Cardiac MDR phenotype, daunomycin, PCR, reversing agents.

Introduction

Resistance of malignant tumors to multiple chemotherapeutic agents is a common cause of treatment failure. One major mechanism of multidrug resistance in mammalian cells involves the increased expression of a 170 kDa plasma membrane glycoprotein named P-glycoprotein (Pgp).[1] This protein acts as an active efflux pump for different cytotoxic drugs including anthracyclines and thus confers the MDR (multidrug resistance) phenotype.[2]

It has been showed that MDR is highly expressed in many clinically resistant tumors and represents an adverse prognostic factor in some cancers.[3,4] However several non-cytotoxic drugs can inhibit the Pgp function and sensitize MDR cells *in vitro*. Combined therapy with MDR-related drugs and MDR inhibitors can decrease tumor size and prolong life span in some animal models.[5]

MDR inhibition or modulation could therefore increase the potency of chemotherapy, but can also unveil new toxicities of anticancer drugs because of the inhibition of the normal protective function of Pgp in various normal tissues or because of a pharmacokinetic interaction between anticancer drugs and MDR inhibitors.[6]

Anthracyclines are very efficient cytotoxic drugs against a broad spectrum of human tumors but they are also an excellent substrate for Pgp. However, their chemotherapeutic use is limited clinically by a well-documented dose-dependent cardiotoxicity.[7] The principal mechanism of daunomycin resistance involves the overexpression of a gene coding for the synthesis of the transmembrane Pgp.[8,9] Schinkel *et al.* have reported a strong increase in the cardiac concentration of vinblastine in homozygous *mdr1a*−/− deficient mice due to drug accumulation and retention in the heart.[10] *In vivo* studies in the SCID mouse have shown that different modulators of MDR and in particular cyclosporin A markedly increased the concentration of doxorubicin in the heart and showed marked multifocal random myocytolysis with myofibrillar disruption in histological heart preparations.[11] Pgp is present in some normal

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tissues,[2,3] it is possible that the combination of such a MDR modulator with a cytotoxic agent might increase or induce normal tissue damage.[1,2] Could the cardiac toxicity of MDR-related drugs such as anthracyclines be correlated to the level of *mdr1* expression in the heart?

Conflicting results have been reported. While Thiebaut *et al.* failed to detect any Pgp in the heart or striated muscle with monoclonal antibody MRK16,[2] Schinkel *et al.* found a significant level of cardiac *mdr1* mRNA detectable by a RNase protection procedure.[10]

The purpose of this work was to try to detect *mdr1a* gene expression in rat heart cells by RT-PCR and, if any, to assess the functional properties of the Pgp by measuring the intracellular accumulation of [^{3}H]daunomycin with and without MDR inhibitors.

Materials and methods

Myocardial cell culture

The cultures were prepared according to the technique of Harary *et al.*[13] with a few modifications. Monolayers of spontaneously beating newborn rat ventricular myocytes were obtained from 2- to 4-day-old Wistar rat hearts. Hearts were trimmed of connective tissue and atria, finely minced, and serially exposed to 0.1% trypsin (Difco, Detroit, MI) for 10 min at 37°C. Gentle trituration and agitation on an orbital shaker bath optimized the enzymatic digestion. Cells released from the first trypsin exposure were discarded and cells obtained from the next nine exposures were then added to an equal volume of trypsin deactivating solution consisting of ice-cold culture medium. Cells were centrifuged at 900 *g* for 5 min and resuspended in Ham F-10 culture medium (Gibco/BRL, Cergy-Pontoise, France), complemented with 14 mM $NaHCO_3$, 1 mM $CaCl_2$, 2 mM glutamine, 6 μg/ml gentamycin, vitamins and 10% fetal calf serum. The suspension was then placed in a culture dish. To reduce the proportion of non-muscular cells, the differential attachment technique was applied.[14] After an attachment phase of 120 min, the final plating of the supernatant was done in 35 mm diameter culture dishes (500 000 cells in 2 ml of nutrient medium).

The cultures were incubated at 37°C in a water-satured atmosphere (5% CO_2, 95% air). By 3 days, confluent monolayers with at least 80% of synchronously beating ventricular myocytes were obtained and used for the experiments.

Nucleic acid preparation and RT-PCR

Total RNA was extracted from frozen adult and newborn rat cardiac tissue, from newborn rat cultured myocardial cells, and from frozen adult rat kidney tissue; this latter was used as a positive control (see below). Total cellular RNA from cells and homogenized tissues was isolated with Trizol reagent (Gibco/BRL) and 1 μg of each RNA was reverse transcribed into cDNA with MMLV RNase H-Reverse Transcriptase (Gibco/BRL).

The Macintosh program Primer 05 (Whitehead Institute for Biomedical Research, Cambridge, MA) was used to design primers that span an intron, thus allowing us to distinguish between cDNA and genomic DNA amplification. The sense primer (5′ CAG TAC ACA TAC CGT CAG CGA CAC) was upstream exon 6 and the antisense primer (5′ ACG TCA AAC CAG CCT ATC TCC TG) inside exon 6 in agreement with the *mdr1a* rat sequence.[15] The primer pair was optimized for annealing temperature (56°C) and $MgCl_2$ concentration (5 mM). The cycle number (42) was optimized so that the DNA amplification did not reach a plateau. After PCR with an automatic DNA thermal cycler (MJ Research, Prolabo, France), we obtained an amplification of a 204 nucleotide product from 390 to 593. Then, 20 μl of each PCR product was run on a 7% polyacrylamide gel and the amplified sequences were visualized with ethidium bromide (Gibco/BRL).

Intracellular retention of [^{3}H]daunomycin

Thirty minutes before the experiment, the incubation medium was replaced by 2 ml of serum-free medium. [^{3}H]Daunomycin (Du Pont de Nemours, Boston, MA) was stored at −20°C and used at a concentration of 50 nM (2.7 Ci/mmol). Because of the uptake of daunomycin reached a steady-state within 15–30 min,[16] the cells were then incubated for 2 h with 50 nM [^{3}H]daunomycin and 50 μM verapamil (Sigma, Saint-Quentin Fallavier, France), 1 μM PSC 833 (Sandoz, Rueil-Malmaison, France) or 10 μM S9788 (Servier, Courbevoie, France). The dishes were rapidly washed three times with isotope-free cold saline in order to block all membrane exchanges. The cells were scraped, withdrawn in 2 ml of PBS solution and sonicated. An aliquot was quantified for protein content by the method of Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Radioactivity was determined in a 1 ml aliquot, using a Packard Tricarb 4530 scintillation spectro-

meter after addition of Ultima-Gold scintillation cocktail (Packard Instrument, Meriden, CT).

Six independent experiments, including 12 dishes each, were carried out. All data points were determined in triplicate with a preparation obtained from the same culture. The net accumulation obtained with verapamil, PSC 833 and S9788 was expressed relative to the value obtained in control conditions on the same day, using the same batch of cells and the same tracer preparation.

Results are expressed as mean ± SD. Comparisons between several groups were made using non-parametric tests: Kruskal–Wallis *H* and Mann–Whitney *U*.

Results

RT-PCR analysis

Based on the fact that some normal human tissues such as kidney, colon, liver and adrenal have a higher expression of the *mdr1* gene than other tissues,[5] we have used kidney as a positive control for detection of *mdr1a* transcripts in cardiac tissue and cultured myocardial cells. After RT-PCR, samples were run on a 7% polyacrylamide gel and the amplified sequence was visualized at 204 nucleotides with ethidium bromide (Figure 1). Ethidium bromide revealed an easily detectable 204 nucleotide band of the *mdr1a* amplified fragment in the different samples. However, the signal appeared stronger in kidney tissue and cultured myocardial cells than in cardiac tissue.

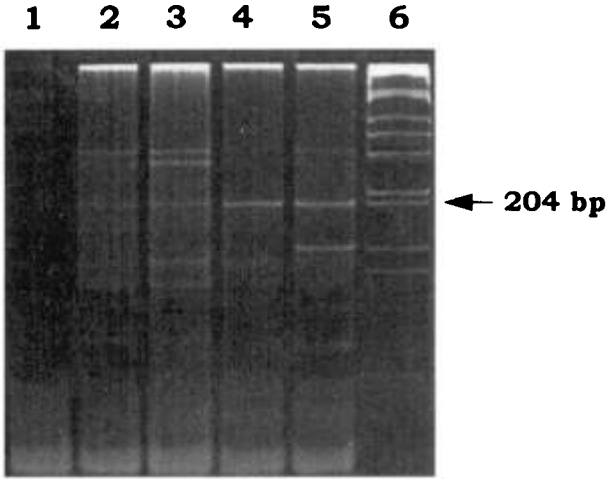

Figure 1. Ethidium bromide-stained polyacrylamide gel of PCR products. PCR products (20 μl) of negative control (1), adult (2) and newborn (3) rat heart, newborn rat cardiac myocytes (4) and kidney (5) were loaded next to a molecular weight DNA marker (6) (Gibco/BRL). The size of the *mdr1a* fragment (in bp) is indicated by an arrow. The 204 bp fragment is present in lanes 2, 3, 4 and 5.

Intracellular retention of [^{3}H]daunomycin

In our experimental conditions, the intracellular concentration of daunomycin measured after a 120 min incubation was 85.5 fmol/μg protein. Thus, this anthracycline exhibited a strong concentration in cells, illustrated by an intracellular to extracellular concentration ratio (C_i/C_e) of 2890. When myocardial cells were incubated with daunomycin and a MDR reversing agent (verapamil, PSC 833 or S9788), we observed a moderate but significant ($p < 0.05$) increase of the intracellular [^{3}H]daunomycin concentration (Table 1).

Discussion

It appeared of great interest to assess MDR expression in heart tissue since MDR reversing agents are associated with anthracyclines in clinical trials.

Our aim was to study *mdr1a* gene expression and Pgp in rat cardiac preparations and to evaluate the functional state of Pgp by using drugs known for their reversing effects.

Table 1. Net cellular accumulation of [^{3}H]daunomycin in myocardial cells after 120 min incubation: effects of addition of verapamil (50 μM), PSC 833 (1 μM) or S9788 (10 μM)

	Control ($n = 18$)	Verapamil ($n = 18$)	PSC 833 ($n = 18$)	S9788 ($n = 18$)
Mean ± SD (% of control values)	100 ± 9.5	108 ± 12[a]	105 ± 11.7[a]	115 ± 21.5[a]

Results from six independent experiments.
[a] $p < 0.05$ when compared to control by Mann Whitney *U*-test.

In heart, the MDR phenotype has already been assessed in normal human tissue by immunohistochemistry[2] and in mouse by the RNase protection procedure.[10] We used a RT-PCR procedure, a rapid, convenient, specific and sensitive technique for the detection of *mdr1a* gene expression. Our results showed a *mdr1a* gene specific expression in all cardiac cells and tissue preparations. In spite of not being able to quantify the expression of *mdr1a* with our technique, it was noticed that the signal was stronger in cultured cells than in cardiac tissue with the same amount of RNA. This obvious difference of intensity leads us to assume that *mdr1a* cardiac expression is more specifically localized into myocytes.

Because *mdr1a* gene expression was detected in the heart, we tried to detect, by immunohistochemistry, the presence of Pgp in this tissue. We used a triple-layer immunoperoxidase technique and the C219 monoclonal antibody (Centocor, Malvern, PA), that is the only available monoclonal antibody specific for rat Pgp.[17] A strong diffuse staining with C219 monoclonal antibody was obtained, suggesting a non-specific labeling. This result is not surprising since Thiebaut *et al.* have reported that C219 monoclonal antibody can react with the heavy chain of the muscle isoform of the myosin.[18] Consequently the detection of Pgp with C219 monoclonal antibody in the heart is not sufficient to demonstrate the presence of Pgp in rat cardiac tissue.

This non-conclusive result led us to search for indirect evidence of Pgp presence using a functional approach. This was investigated by evaluating the accumulation of [^{3}H]daunomycin in cell cultures with and without inhibition of Pgp. Our results showed an important accumulation of [^{3}H]daunomycin in myocytes, as also reported in wild-type murine tumor cell lines.[16] This is in agreement with the high capacity of daunomycin to bind phospholipids,[19,20] nucleic acids,[21] lysosomes[22] and other cellular components, so that the majority of the intracellular drug is in a bound form.[23]

This high intracellular concentration of daunomycin in myocytes is slightly but significantly increased in the presence of MDR inhibitors. This result could be compatible with the expression of the *mdr1a* gene and the presence, at a low level, of a functional Pgp in normal cardiac cells; consequently, the difference observed between controls and treated cells could be due to a decrease of efflux and not to an increased uptake.

Many authors agree that the penetration of daunomycin into the cells is compatible with a mechanism of passive diffusion.[22–24] The increased accumulation of daunomycin in the presence of metabolic inhibitors suggests that the fibroblasts[22] and various tumor cell lines, sensitive or resistant,[16,25] have an active efflux mechanism. This efflux mechanism is more efficient in resistant cell lines. However, recent results suggest that the efflux mechanism in sensitive cells involves only passive diffusion of the neutral form of the drug, while in resistant cells, they propose the coexistence of two types of efflux: passive diffusion and energy-dependent Pgp-related mechanism.[24]

The existence of an energy-dependent mechanism of efflux may justify the slightly higher accumulation of daunomycin in cells in the presence of S9788, since S9788 has been reported to be an uncoupler of oxidative phosphorylation.[26]

Finally, our results can be summarized as follows: (i) a high concentration of [^{3}H]daunomycin into myocardial cell cultures may be linked to the clinical cardiotoxicity of anthracyclines, (ii) direct evidence of *mdr1a* gene expression and (iii) indirect arguments for a moderate but significant Pgp function in rat heart.

It has been proposed that Pgp plays a role in the protection of the organism against toxic xenobiotics, by active excretion of these compounds into bile, urine or intestinal lumen, and by preventing or limiting accumulation in critical organs such as testis, brain and heart.[2,10] In clinical studies, increased toxicities have been observed for etoposide, daunomycin and doxorubicin when they were combined concurrently with verapamil or cyclosporin.[6] Our results, as with those of Schinkel *et al.* concerning the cardiac toxicity of MDR-related drugs in *mdr1a*−/− mice,[10] ask the question about the importance of a moderate Pgp function in organ protection and imply that we need to be careful in the clinical use of MDR reversing agents.

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