Cell-specific effects of RAS oncogene and protein kinase C agonist TPA on P-glycoprotein function

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Abstract **We compared the influence of exogenous N-*ras* oncogene and treatment with PKC agonist 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on P-glycoprotein (Pgp) function in various human, rat and dog cell lines. Two approaches were used: (a) flow cytometry analysis of Rhodamine 123 (Rh123) exclusion; and (b) sensitivity to cytotoxic action of colchicine. We have found that in Rat1 fibroblasts, rat IAR2 epithelial cells and rat McA RH 7777 (hepatoma), *ras* activates Pgp function, while in MDCK (dog kidney), K562 (human chronic myelogenous leukaemia) and LIM1215 (human colon carcinoma) cells it either has no effect or even acts in opposite direction. TPA-induced Pgp function shows dissimilar pattern of cell specificity. It is assumed that PKC and *ras* oncogene regulate *mdr1* gene expression through at least partially distinct signalling pathways.**

Key words: RAS oncogene; Protein kinase C; Multidrug resistance, P-glycoprotein

1. Introduction

Acquisition of MDR in vitro and in vivo usually results from increased expression of the *mdr1* (*pgy1*) gene encoding so-called Pgp, the 170–180 kDa transmembrane glycoprotein that functions as an energy-dependent efflux pump for various lipophilic compounds [1–3]. A lack of response of human tumors to chemotherapy is often due to increased *mdr1* gene transcription [2]. The regulation of *mdr1* gene activity is studied poorly. Recently it was shown that PKC-activating agents induce *mdr1* expression and as a result enhance Pgp function [4]. Also, it was found that *ras* oncogenes are able to activate a reporter gene expression driven by human *mdr1* gene promoter in murine immortalized fibroblasts [5]. However, the functional significance of this finding was unclear. Meanwhile an understanding the role of *ras*-mediated signalling pathway in regulation of *mdr1* gene activity might be important for prediction of response to therapy with definite drugs since activation of *ras* genes is characteristic of many types of human tumors [6]. To study whether activated *ras* oncogene is really able to induce Pgp-mediated MDR we created the retroviral construct expressing human N-ras^{asp12} cDNA, introduced it into various human and rodent cell lines and analyzed the changes in Pgp function in the resultant cells. In addition, in order to elucidate whether the same signalling pathway might be responsible for *ras*- and PKC-induced MDR we compared the effects of exogenous *ras* and TPA treatment in cells of different origin.

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Abbreviations: MDR, multidrug resistance; FCM, flow cytometry; Pgp, P-glycoprotein; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PBS, phosphate-buffered saline; Rh123, Rhodamine 123; FCS, fetal calf serum; PCR, polymerase chain reaction.

2. Materials and methods

2.1. Cell lines

LIM1215 (human colon carcinoma) [7], K562 (human chronic myelogenous leukaemia) [8], MDCK (dog kidney cells) [9], McA RH 7777 (rat hepatoma) [10], IAR2 (minimally transformed rat epithelial cells) [11] and Rat1 (immortalized fibroblasts) were used. The cells were cultivated in DMEM or RPMI 1640 (for LIM1215, K562 and McA RH 7777) media supplemented with 10% of FCS.

2.2. Expression vectors; isolation of cell sublines expressing exogenous RAS

In order to transfer *RAS* oncogene into different cell lines, including those already expressing plasmids with *neo* gene, we prepared on the basis of pPS retroviral vector [12] the construct containing human activated N-ras^{asp12} and selectable *hygro* (resistance to hygromycin) genes. The N-ras^{asp12} cDNA [13] was subcloned as BamHI-HindIII fragment from the pBS-ras plasmid (provided by Dr. A. Tatosyan, Cancer Research Center, Moscow) into appropriate cloning sites of pPS-3/hygro vector. The resulting construct was introduced by Ca^{2+}P-transfection into murine amphitropic packaging ψCRIP [14] cells. After selection with hygromycin (200 μg/ml for three weeks) the obtained clones were combined and used as producers of recombinant pPS/hygro-ras virions. The culture medium in which these cells (about 10^5 per ml) were cultivated for one day was filtered through 0.45 μm membranes (Millipore), diluted (1:3–1:5) by medium containing 1% of FCS and 8 μg/ml of polybrene (Serva), and added to LIM1215, K562, McA RH 7777, IAR2, Rat1 or MDCK cells (1 ml of mixture per 10^5 cells). The next day it was changed for fresh growth medium. After additional cultivation for 24–48 h the cells were plated 1:5 or 1:10 onto 60 mm dishes containing selective media (hygromycin, 150–250 μg/ml). After 2–3 weeks with weekly medium change the resulting resistant cultures were trypsinised and replated. To minimise the influence of clonal variability we developed from all parental cell lines 1–2 independent sublines each derived from dozens of individual clones.

The cultures were tested for expression of introduced N-*ras* by cDNA-PCR analysis. For this purpose total cell RNA was isolated by guanidine thiocyanate extraction. Synthesis of cDNA specific for recombinant construct was performed on RNA template by using 24 bp primers that anneal just downstream of pPS/hygro cloning sites. The reaction mixture containing 15 pmol of primers, 1 μg of RNA and 5 units of TET-Z Tth DNA-polymerase (BioMaster, Moscow) in 20 μl of reverse transcription buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM tris.HCl pH 8.8, 1 mM $MnCl_2$, 2.5% glycerol, 200 μM each of dATP, dGTP, dCTP and dTTP] was incubated for 15 min at 70°C. Aliquots (2–5 μl) from reverse transcription reaction were then used as DNA templates for PCR reaction using a pair of 24 bp primers specific to the regions of pPS/hygro flanking cloning site.

Changes in morphology and growth parameters of Rat1 cell sublines transformed by pPS/hygro-ras were described by us earlier [15].

2.3. FCM analysis of Pgp function

Evaluation of Rh123 efflux from various cells was performed ac-

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cording to the described procedures [16,17]. The cells were detached from plastic, incubated with 5 mg/ml of Rh123 (Sigma) for 10 min at 37°C, washed twice with PBS and incubated for 30 min in dye-free medium. The cell fluorescence was evaluated by the FACScan flow cytometer (Becton-Dickinson).

2.4. Sensitivity to cytotoxic drugs

This was determined by colony formation assay or cell counting after cultivation in media containing different concentrations of colchicine (Merck).

3. Results and discussion

Rat1 cell derivatives transformed by pPS/hygro-ras construct showed significant increase in resistance to colchicine (Fig. 1). The LD_{50} (drug dosage diminishing the number of clonogenic cells by half) for all independently selected sublines that express exogenous N-*ras* was increased 3 to 4.5-fold as compared with parental Rat1 or Rat/neo cells (Table 1). FCM analysis of efflux of Rh123, a Pgp-transported fluorescent mitochondrial dye, showed that in Rat1 sublines expressing N-*ras* the proportion of Rh123-dull cells is higher than that in the parental cells. Pgp inhibitor verapamil abolished *ras*-induced stimulation of Rh123 efflux (Fig. 2). So, in agreement with the data showing activation of *mdr1* gene promoter by *ras* oncogene [5] we observed in *ras*-transformed Rat1 cell sublines stimulation of Pgp function. However several other cell lines (MDCK dog kidney, K562 human myelogenous leukaemia, and LIM1215 human colon carcinoma cells) expressing the same pPS/hygro-ras retroviral construct showed neither decreased colchicine sensitivity, nor enhanced Rh123 exclusion (Figs. 1,2; Table 1). Meanwhile, according to the data of cDNA-PCR analysis (Fig. 3) the levels of exogenous *ras* mRNA in these cells were comparable with those found in rat cell lines showing either pronounced (Rat/neo fibroblasts; IAR2 rat epithelial cells) or

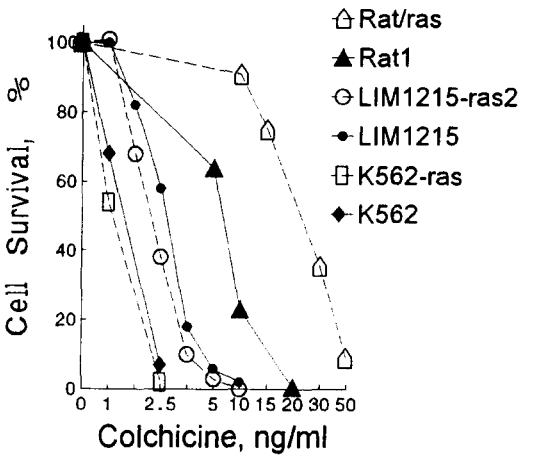

Fig. 1. Influence of expression of exogenous N-ras^{asp12} on sensitivity of various cell lines to colchicine. Cell survival was estimated as a ratio of a number of colonies (for Rat1 sublines) or cells (for K562 and LIM1215 sublines) grown at given colchicine dosage to those grown in drug-free medium. For clonogenic assay 3×10^2 cells were seeded in duplicate onto 60 mm dishes for 14 days. In cell yield assay 5×10^4 cells were plated into 25 cm^2 flasks for 7 days. For each pair of cell sublines the data of one of typical experiments are presented.

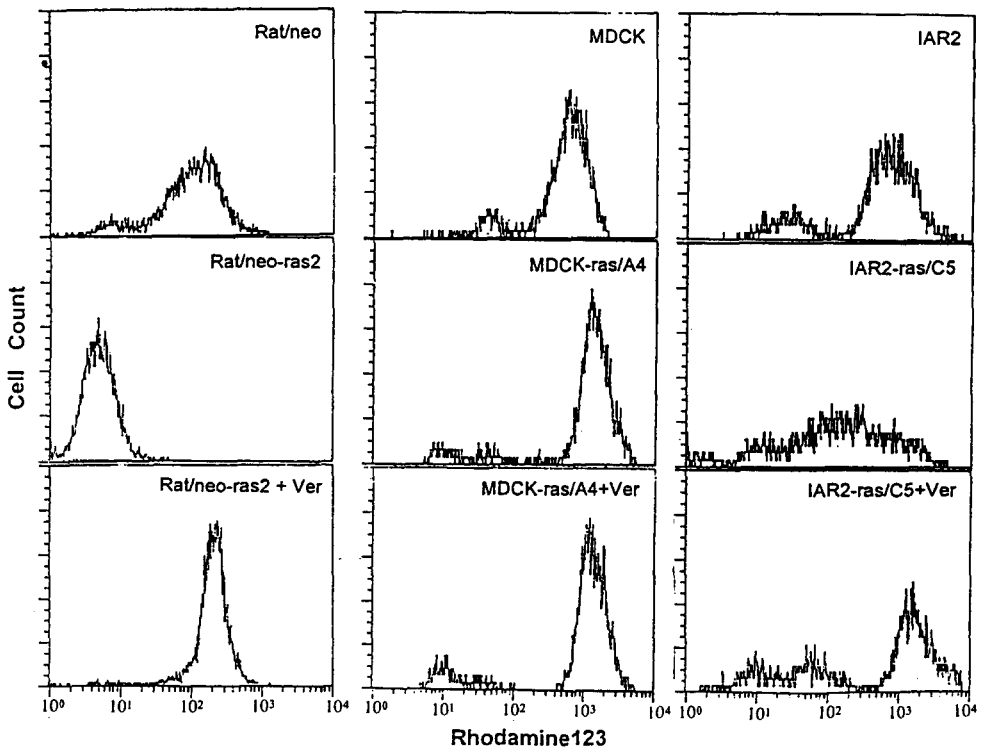

Fig. 2. Effects of N-*ras* oncogene on Pgp activity in Rat1, MDCK, and IAR2 cells. Logarithmic fluorescence intensity of *ras*-expressing and parental was studied after staining with Rh123 as described in section 2. N-*ras* stimulated Rh123 efflux in Rat/neo-ras2 and IAR2-ras/C5 cells, while in MDCK-ras/A4 its expression was accompanied by some inhibition of Pgp activity. Incubation in the presence of 30 mM of verapamil (the lower row) caused complete reversion of *ras*-induced activation of Pgp function in Rat1 and IAR2 cell derivatives whereas in MDCK-ras/A4 cells it did not influence Rh123 efflux.

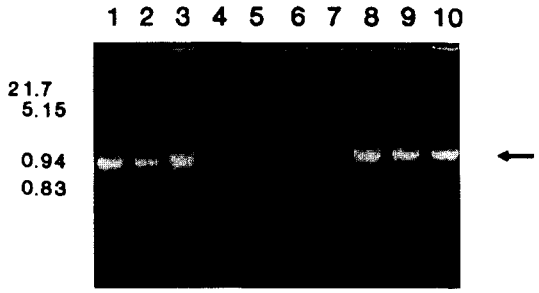

Fig. 3. cDNA-PCR analysis of cell sublines developed as a result of retrovirus mediated transfer of pPS/hygro-ras construct. cDNAs specific for mRNAs produced by exogenous constructs were synthesized and amplified as described in section 2, separated on 1% agarose gel and visualized by ethidium bromide staining. In all the sublines selected with hygromycin after introduction of pPS/hygro-ras (tracks 1–3, 7–10 – K562-ras, IAR2-ras/F10, LIM1215-ras1, MDCK-ras/B2, IAR2-ras/C5, MDCK-ras/C4, and Rat/neo-ras1, respectively) the fragment of expected molecular weight (about 900 bp) is seen (arrow). In Rat/hygro cells (track 6) infected with insert-free pPS/hygro construct a low-molecular weight fragment (about 300 bp) corresponding to the vector adapter region was amplified. Parental K562 (track 4) and LIM1215 (track 5) do not show the pPS-specific amplification products. Left the position of the marker fragments is indicated.

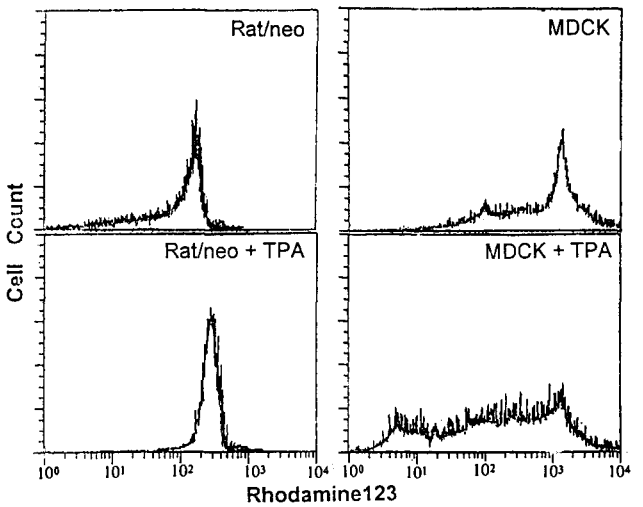

Fig. 4. Differential effects of TPA and activated *RAS* on Pgp activity in Rat1 and MDCK cells. Unlike N-ras^{asp12} TPA stimulates Rh123 efflux in MDCK but not in Rat/neo cells where it causes slight inhibition of Pgp function.

slight (McA RH 7777 rat hepatoma) induction of Pgp function (Table 1). Such differences in activation of Pgp function may reflect either species-specific (rodent vs. dog and human), or tissue-specific distinctions in response to *ras* oncogene. In previous studies cell-specific stimulation of *mdr1* gene expression was observed after treatment with cytotoxic drugs [18] and PKC agonists [4]. Interestingly that while the former study revealed some distinctions between human and rodent cells, the latter observed differences in *mdr1* gene response in various cell lines of human origin. The results obtained to date are insufficient to understand the basis of differential susceptibility of various cell lines to *ras*-mediated stimulation of Pgp activity. However they indicate that *ras* gene mutations may have different prognostic significance in prediction of response to chemotherapy of various types of human tumors.

In cells transformed by *ras* oncogenes activation of PKC is often observed [19]. To elucidate whether the same signalling pathway might be responsible for *ras*- and PKC-induced activation of *mdr1* gene we compared the effects of exogenous N-ras^{asp12} and TPA (an efficient activator of PKC) on Pgp function in a variety of cell types. We found that majority of cell lines tested showed differential sensitivity to Pgp-related effects of *ras* and TPA. For example, expression of N-ras^{asp12} in MDCK cells causes no increase in Pgp function (Fig. 2) while treatment of MDCK cells with TPA increases the proportion of Rh123-dull cells (Fig. 4). Differential response of Pgp-mediated Rh123 efflux to *ras* and TPA was observed also in Rat1 and LIM1215 cell lines (Figs. 2,4; Table 2). These findings suggest that *ras* oncogenes and PKC probably regulate *mdr1* gene expression through different (at least in part) signalling pathways. The detailed mechanisms of such regulation are a subject for future studies.

Table 1
Cell-specific induction of P-glycoprotein-mediated MDR by activated N-*ras* oncogene

Cells	pPS/hygro-ras expression	Relative drug-resistance*	Rh123 exclusion
Rat1	–	1.0	+
Rat/ras	+	3.1	+++
Rat/neo	–	1.0	+
Rat/neo-ras1	+	4.5	++++
Rat/neo-ras2	+	4.0	++++
IAR2	–	1.0	+
IAR2-ras/C5	+	n.d.	+++
McA RH 7777	–	1.0	+
McA RH 7777-ras2	+	2.1	++
MDCK	–	1.0	+
MDCK-ras/B2	+	n.d.	–
MDCK-ras/A4	+	n.d.	–
K562	–	1.0	+
K562-ras	+	0.7	–
LIM1215	–	1.0	+
LIM1215-ras1	+	0.8	–
LIM1215-ras2	+	0.8	–

*Relation of LD_{50} of colchicine for given cell subline to LD_{50} for parental cells.

Table 2
Comparison of the effects of exogenous N-*ras* oncogene and TPA in various cell lines

Cell line	Stimulation of Rh123 efflux	
	N-*ras*	TPA
Rat/neo	++++	–
McA RH 7777	+	++
MDCK	–	+
LIM1215	–	+

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