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FEBS Letters 341 (1994) 295–298

FEBS 13804

FEBS LETTERS

Partial restoration of the actin cytoskeleton in transformed Syrian hamster fibroblasts selected for low levels of 'typical' multidrug resistance

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Received 3 February 1994

Abstract

Two independent colchicine (CLC)-resistant sublines of Rous sarcoma virus-transformed Syrian hamster fibroblasts were isolated. Each subline represented variants with 11- and 12.4-fold resistance, respectively, their 23- and 23.7-fold resistant descendants, as well as variants cultured in CLC-free medium for 10 months without loss of resistance. All variants demonstrated 'typical' multidrug resistance. The parental cells contained actin in dispersed form, as determined by rhodamine-phalloidin staining. In contrast, already in 11- and 12.4-fold resistant sublines up to 30% of cells demonstrated restored stress fibers. Cultivation in CLC-free medium leads to the accumulation of cells with a partially restored actin cytoskeleton. Putative mechanisms of up-regulation of stress fiber assembly in cells with P-glycoprotein-mediated multidrug resistance are discussed.

Key words: Actin; Multidrug resistance; P-glycoprotein; Syrian hamster fibroblast

1. Introduction

Multidrug resistance is one of the main reasons for chemotherapeutic failures in cancer patients. 'Typical' MDR (T-MDR) is characterized by the following phenomena: (i) cells selected for insensitivity to a single drug demonstrate cross-resistance to certain agents; (ii) intracellular drug accumulation is decreased; (iii) 140–170 kDa transmembrane Pgp is overexpressed [1]. The Pgp-mediated efflux of cytostatics is considered to be the major mechanism that defines T-MDR.

The evolution of T-MDR is accompanied by significant alterations of cellular morphology and physiology, e.g. changes in cell spreading, growth rate, membrane traffic, decrease of tumorigenicity and metastatic capability, etc. [2–5]. These characteristics of cell behavior are mediated, at least in part, by the actin cytoskeleton (see [6,7] for reviews). Partial restoration of the actin cytoskeleton in cells with a high level of drug resistance have been shown previously [5]. However, phenotypic alterations may already arise in cells with low levels of drug resistance; so, we suggested that certain changes of the actin cytoskeleton might emerge just at the early steps of selection for T-MDR.

2. Materials and methods

2.1. Cell lines

The HET-SR-2SC-LNM line, the Syrian hamster embryo fibroblasts transformed by RSV, Schmidt–Ruppin strain, was provided by Prof. G. Deichman, Cancer Research Center, Moscow. This line was used as the parental line for the isolation of drug-resistant variants. Cells were propagated in RPMI-1640 plus Dulbecco's modified Eagle's medium (1:1, v/v) supplemented with 5% fetal calf serum (Flow), 2 mM L-glutamine and 50 U/ml gentamycin (complete medium). For the isolation of sublines with T-MDR, the parental cells were continuously treated with increasing concentrations of CLC (Merck, Germany). CLC-resistant sublines were propagated in complete medium supplemented with CLC.

2.2. Cell viability in the presence of cytostatic drugs

Cell viability in the presence of cytostatic drugs was studied in an MTT-assay as described elsewhere [8]. The drugs used were CLC, vinblastine (G. Richter, Hungary); Farmorubicin (Farmitalia Carlo Erba, Italy); methotrexate and Platidiam (Lachema, Czechoslovakia). IC_{50}, 50% growth inhibition concentration, and resistance levels were calculated as indicated in legend to Table 1.

2.3. In vitro drug uptake

5×10^5 cells/ml were plated onto 24-well plates (Costar) and incubated overnight at 37°C, 5% CO_2, [^{3}H]Vincristine (10^{-6} M, 0.037 MBk; Amersham) or Farmorubicin (10^{-6} M) were added. Parallel wells were coincubated with drug and T-MDR-reversing agent Finoptin (5×10^{-6} M, Orion). After 30 min incubated cell monolayers were washed 3 times with cold PBS, pH 7.2. For the study of farmorubicin accumulation, cells were lysed in 0.3 N HCl in 50% ethanol and spun ($100 \times g$, 30 min). Supernatants were assayed with a Hitachi spectrofluorimeter (exitation 470 nm, emission 585 nm) [9]. Drug content was estimated using standard calibrating curves. For the study of [^{3}H]vincristine uptake, cells

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Abbreviations: CLC, colchicine; PAGE, polyacrilamide gel electrophoresis; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; RSV, Rous sarcoma virus; SDS, sodium dodecyl sulfate; T-MDR, 'typical' multidrug resistance.

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SSDI 0014-5793(94)00189-3

were lysed in 0.5 N NaOH overnight at 37°C and radioactivity of the samples was measured with a β-counter (LKB). Protein concentrations were measured by the method of Lowry [10].

2.4. Western blot detection of Pgp

Cells were detached from culture plastic, pelleted and lysed in buffer (10% sucrose; 62.5 mM Tris, pH 6.8; 2 mM EDTA; 2% SDS; 2 mM PMSF). Further steps of lysate preparation and electroblotting were performed as described [11]. Nitrocellulose filters were probed with murine monoclonal antibodies C219 (Sighet Lab. Inc., a gift from Prof. M. Dietel, Christian-Albrecht's Universität, Kiel). Bound antibodies were detected, using rabbit anti-mouse antibodies coupled with peroxidase and a substrate mixture containing 4-chloro-naphtol (Sigma).

2.5. Immunocytochemistry

Rhodamine-phalloidin staining of F-actin was carried out exactly as described [12]. Cells were examined under a Photomicroscope 3 (Opton, Germany) and photographed. 500 cells of each subline were examined in different fields of coverslips, and the percentage of cells demonstrating stress fibers was calculated.

3. Results

The selective procedure and drug resistance levels of cells used in this study are shown in Table 1. Two independent CLC-resistant sublines (variants with 11; 12.4 and 23; 23.7-fold resistance to CLC) were isolated in vitro from the wild type HET-SR-2SC-LNM cell line by step-wise selection. In addition, 2SC/20R-1 and 2SC/20R-2 sublines were maintained; these derivatives of 2SC/20-1 and 2SC/20-2 sublines, respectively, were cultured in CLC-free medium for 10 months but did not lose CLC-resistance (Table 1).

Table 1
Isolation of CLC-resistant sublines from HET-SR-2SC-LNM line

Cell line	Origin	Duration of CLC treatment (weeks)*	IC_{50} (μg/ml)
HET-SR-2SC-LNM (parental)	–	–	0.36 ± 0.03 (1)
2SC/4-1	selected from parental at 4 μg/ml	3	4.47 ± 0.47 (12.4)
2SC/4-2	selected from parental at 4 μg/ml	5	3.95 ± 0.39 (11)
2SC/20-1	selected from 2SC/4-1 at 20 μg/ml	6	8.27 ± 0.27 (23.0)
2SC/20-2	selected from 2SC/4-2 at 20 μg/ml	6	8.55 ± 0.09 (23.7)
2SC/20R-1	2SC/20-1 propagated in CLC-free medium for 10 months	6	8.15 ± 0.11 (22.6)
2SC/20R-2	2SC/20-2 propagated in CLC-free medium for 10 months	6	8.20 ± 0.09 (22.8)

IC_{50}, a 50% growth inhibition concentration, mean ± S.E. from 3 experiments.
*After step-wise selection all CLC-resistant sublines were propagated in complete medium containing CLC. The drug was omitted from the medium 1–2 passages prior to the determination of IC_{50}.
Resistance levels $\left(\frac{IC_{50} \text{ of resistant subline}}{IC_{50} \text{ of parental line}}\right)$ are given in parentheses.

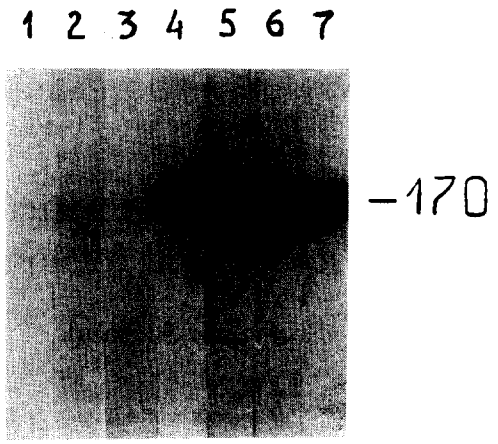

Fig. 1. Western blot assay of P-glycoprotein. Cell lysates were resolved by SDS-PAGE, electroblotted and probed with C219 antibodies directed to P-glycoprotein. Lanes 1, HET-SR-2SC-LNM; 2, 2SC/4-1; 3, 2SC/4-2; 4, 2SC/20-1; 5, 2SC/20-2; 6, 2SC/20R-1; 7, 2SC/20R-2. The molecular weight (in kDa) is indicated at the right of the panel.

Pgp was clearly detected in 2SC/20-1 and 2SC/20-2 cells as well as in both 2SC/20R sublines (Fig. 1), as detected by Western blotting with anti-Pgp antibodies. The resistant sublines demonstrated cross-resistance to vinblastine, Farmorubicin, but not to methotrexate or Platidiam (Table 2). The uptake of MDR-related drugs, ^{3}H-vincristine and farmorubicin, in 2SC/20-1 and 2SC/20-2 sublines was significantly lower than in parental cells, and was finoptin-reversible (Table 3). Taken together, these data indicate that the selected sublines demonstrated characteristic patterns of T-MDR.

We used the rhodamine-phalloidin staining to study the actin cytoskeleton in wild type (drug-sensitive) cells and their variants with T-MDR. Parental HET-SR-2SC-LNM fibroblasts shared intensive diffuse cytoplasmic staining (Fig. 2A,B). We failed to visualize stress fibers in parental cells. In contrast, 25–30% of the variants in the 2SC/4-1, 2SC/4-2 and 2SC/20-1, 2SC/20-2 sublines demonstrated microfilamentous bundles crossing the cell (Fig. 2C,D).

Table 2
IC_{50} (ng/ml) and cross-resistance levels (in parentheses) of HET-SR-2SC-LNM cells and CLC-resistant variants

Cell line	Drugs			
	Farmorubicin	Vinblastine	Methotrexate	Platidiam
HET-SR-2SC-LNM	125	5	31	700
2SC/20-1	2875 (23)	135 (27)	22 (0.7)	700 (1)
2SC/20-2	2375 (19)	160 (32)	31 (1)	490 (0.7)

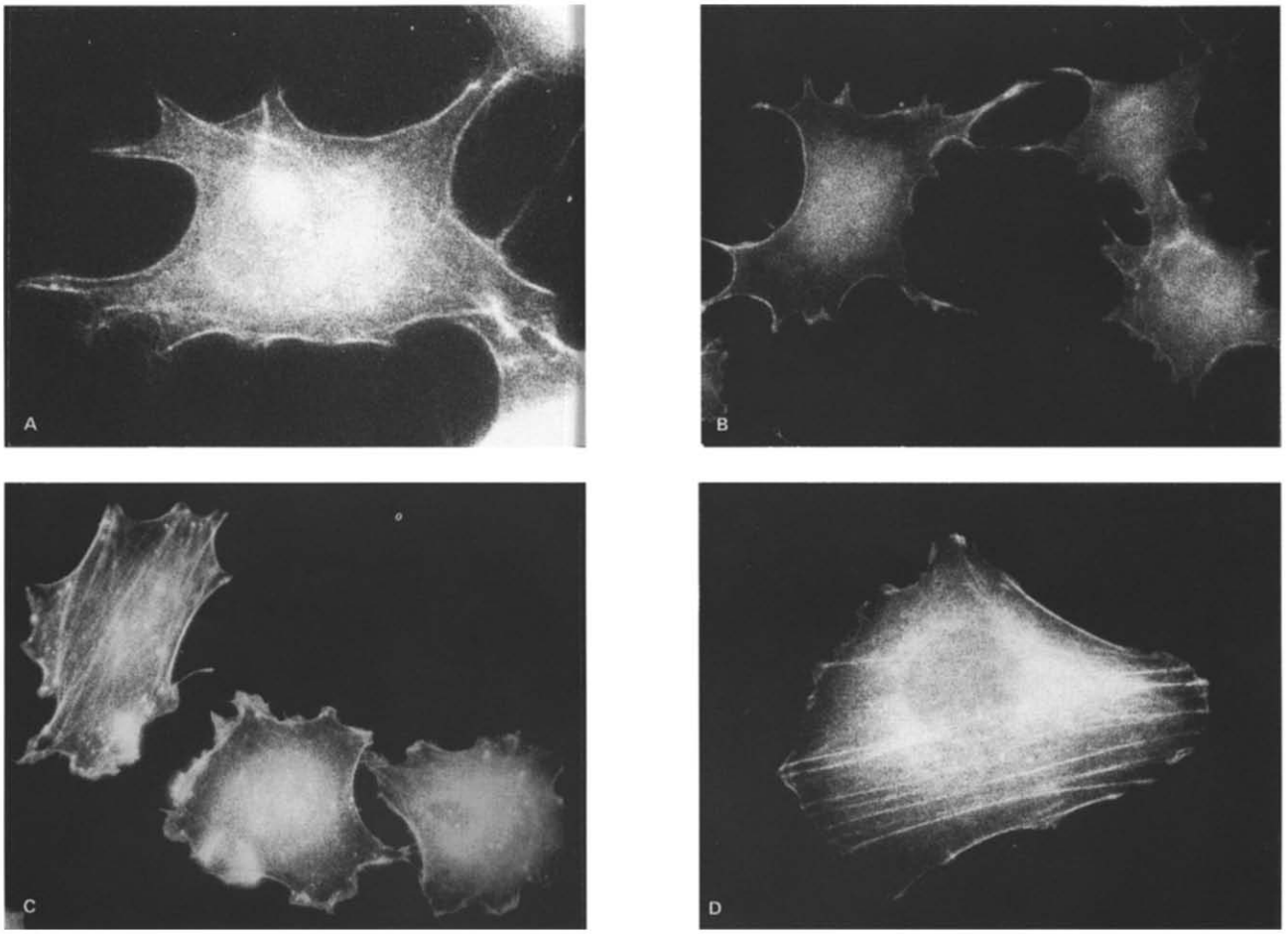

Fig. 2. Rhodamine-phalloidin staining of HET-SR-2SC-LNM cells and sublines with T-MDR. (A,B) HET-SR-2SC-LNM cells, diffuse cytoplasmic staining. (C) 2SC/4-2 subline: cell with stress fibers and variants with diffuse staining; (D) 2SC/20-2 cell, stress fibers crossing the cell. (A,C) × 700; (B,D) × 2100.

In both 2SC/20R-1 and 2SC/20R-2 sublines, cultured in CLC-free medium for a long period without loss of resistance and Pgp overexpression (see Table 1, Fig. 1), the increased number of cells with partially restored actin was found. The majority of cells (more than 50% of the cell population) had clearly visualized stress fibers.

4. Discussion

In the present study we isolated RSV-transformed rodent fibroblasts with low levels of CLC-resistance. These sublines are shown to possess T-MDR, providing evidence that Pgp-mediated drug efflux did confer drug resistance in this experimental model.

Our major result is the tendency for partial restoration of the actin cytoskeleton in cells with initial levels of T-MDR. The data imply that the establishment of Pgp-mediated MDR may be accompanied by the restoration of stress fibers, and these changes are inherited by subsequent cell generations.

Thus, the restoration of the actin cytoskeleton in the course of selection for T-MDR seems to be non-random. The question arises: are there mechanisms common for the up-regulation of both Pgp-mediated drug efflux and actin restoration? We suggest that at least three mechanisms directly associated with the establishment of T-MDR, e.g. the increase in intracellular energy turnover, elevation of the Ca^{2+} influx, and activation of protein kinase C, may account for actin assembly.

First, T-MDR cells need more intracellular ATP, since Pgp-mediated drug efflux requires greater amounts of ATP [13]. On the other hand, ATP is crucial for the elongation of actin monomers [14], and a rapid increase in ATP consumption leads to the restoration of actin [15]. Probably, the increase of energy supply in cells with T-MDR may be the background for the assembly of stress fibers.

Second, the increased Ca^{2+} influx observed in cells with T-MDR [16] may lead to inactivation of capping proteins, thus promoting nucleation [17]. So excess of ATP and Ca^{2+} at the cell periphery could attribute to the initiation of F-actin restoration.

Third, one could propose a phosphatidylinositol 4,5-

Table 3
Uptake of drugs by HET-SR-2SC-LNM cells and 2SC/20 sublines

Cell line	10^{-6} M [^{3}H]Vincristine		10^{-6} M Farmorubicin	
	− Finoptin	+ Finoptin	− Finoptin	+ Finoptin
HET-SR-2SC-LNM	16.1 ± 1.2	22.3 ± 1.1	28.8 ± 1.5	32.2 ± 2.1
2SC/20-1	6.6 ± 0.4[a]	15.7 ± 0.2[b]	9.7 ± 0.6[a]	28.6 ± 1.5[b]
2SC/20-2	7.4 ± 0.1[a]	13.0 ± 0.5[b]	11.0 ± 0.4[a]	27.4 ± 1.3[b]

Cells were incubated either with drug only or with drug and 5×10^{-6} M Finoptin for 30 min, washed, lysed, and the drug content was determined (see section 2). [a]Significantly different (by Student's t-test) from HET-SR-2SC-LNM cells ($P < 0.05$); [b]significantly different from cells incubated without Finoptin ($P < 0.05$).

biphosphate-mediated mechanism to be responsible for a switch of actin assembly in T-MDR cells. Indeed, activation of protein kinase C by phorbol esters or antitumor drugs results in increased Pgp phosphorylation and reduced intracellular drug accumulation, thus providing a direct correlation with drug resistance [18–20]. In turn, phosphatidylinositol 4,5-biphosphate was found to dissociate complexes of profilin and actin [21].

T-MDR cells might utilize stress fibers for a better outward transport of drugs, since disruption of the actin cytoskeleton resulted in loss of the resistant phenotype [22]. These data provide evidence for the biological significance of actin cytoskeleton restoration in T-MDR cells.

Partial restoration of actin cables may reflect changes in signal transduction in T-MDR cells. This might be an attractive explanation for the numerous phenotypic alterations coinciding with the establishment of multidrug resistance.

Acknowledgements: The authors are grateful to Prof. G. Deichman for providing the HET-SR-2SC-LNM cell line, to Drs. I. Tint and T. Svitkina for critical discussions of the manuscript. This study was supported by Russian Academy of Sciences Foundation for Fundamental Research.

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