J. Physiol. Biochem., **56** (4), 307-312, 2000

P-glycoprotein, glutathione and glutathione S-transferase increase in a colon carcinoma cell line by colchicine

M. J. Ruiz-Gómez, A. Souviron, M. Martínez-Morillo and L. Gil

Departamento de Radiología y Medicina Física, Facultad de Medicina, Universidad de Málaga, Teatinos s/n, 29071 Málaga (Spain)

(Received on May 3, 2000)

M. J. RUIZ-GÓMEZ, A. SOUVIRON, M. MARTÍNEZ-MORILLO and L. GIL. *P-glycoprotein, glutathione and glutathione S-transferase increase in a colon carcinoma cell line by colchicine.* J. Physiol. Biochem., **56** (4), 307-312, 2000.

The acquisition of resistance to anticancer agents used in chemotherapy is the main cause of treatment failure in malignant disorders, provoking tumours to become resistant during treatment, although they initially respond to it. The main multidrug resistance (MDR) mechanism in tumour cells is the expression of P-glycoprotein (P-gly), that acts as an ATP-dependent active efflux pump of chemotherapeutic agents. Furthermore, an increased detoxification of compounds mediated by high levels of glutathione (GSH) and glutathione S-transferase (GST), has been found in resistant cells. We developed a study aiming to evaluate the evolution of the main drug resistance markers in tumour cells: P-gly, GSH and GST, during the acquisition of resistance to colchicine, for the purpose of studying the adaptation process and its contribution to the MDR phenomenon. A human colon adenocarcinoma cell line was exposed to colchicine during 82 days, being P-gly, GSH levels and GST activity evaluated by flow cytometry, spectrofluorimetry and spectrophotometry, during exposure time. P-gly and GSH levels increased gradually during the exposure to colchicine, reaching 2.35 and 3.21 fold each. On day 82, GST activity increased 1.84 fold at the end of the exposure period. Moreover, an increment in drug cross-resistance was obtained that ranges from 2.62 to 5.22 fold for colchicine, vinblastine, vincristine and mitomycin C. The increments obtained in P-gly, GSH and GST could probably contribute to the MDR phenomenon in this human colon adenocarcinoma cell line.

Keywords: Flow cytometry, Glutathione (GSH), Glutathione S-transferase (GST), Human colon adenocarcinoma, Multidrug resistance (MDR), P-glycoprotein.

Correspondence to M. Martínez-Morillo (Tel.: 34 952 131 631; Fax: 34 952 131 630; e-mail: mmorillo@uma.es).

The acquisition of resistance to anticancer agents used in chemotherapy is the main cause of treatment failure in malignant disorders, causing tumours to become resistant during treatment, although they initially respond to it (9). Investigations made after obtaining drug resistant tumours by the selection of cell lines capable of growing in high doses of cytotoxic agents such as colchicine, vincristine, vinblastine, etc, have revealed the expression of the multidrug resistant (MDR) phenotype (19). One of the principle mechanisms in MDR is caused by the expression of P-glycoprotein (P-gly), encoded by the multidrug resistance gene (MDR1). It acts as an ATP-dependent active efflux pump of chemotherapeutic agents (5). Furthermore, an increased detoxification of compounds mediated by high levels of glutathione (GSH) and glutathione S-transferase (GST), has been found in resistant cells (12, 23). These molecules are believed to contribute to the resistant phenotype as well (14).

The aim of this study was to investigate whether the resistance mechanisms P-gly, GSH and GST are altered during the acquisition of resistance of a human colon adenocarcinoma cell line to colchicine and the evolution of their levels during the exposition time, for the purpose of studying the adaptation process and its contribution to the MDR phenomenon.

Materials and Methods

Cell culture.– Human colon adenocarcinoma cells (HCA) (13) (cell line provided from Biochemistry Department, Faculty of Medicine, Málaga) were grown in monolayer in modified *RPMI-1640* (Sigma) medium (with L-glutamine, calcium and magnesium free), and supplemented with hepes buffer 1 M (15 ml/L), sodium bicarbonate 7.5 % (28 ml/L), 10 % heat inactivated calf serum and 1 % antibiotic-antimycotic solution 100 X (PSF, Gibco); at 37 °C in 5 % CO_2/air atmosphere.

A colchicine-resistant subline was obtained from HCA cells by continuous exposure of exponentially growing cell cultures to 0.5 µg/ml colchicine (CCH, MERCK, Darmstadt) (15). The selection was performed with CCH because this drug has been widely used to obtain primary resistant cells capable of growing in high drug concentrations (19). After monolayer trypsinization, 5 x 10^5 cells were seeded in Falcon flasks in a medium containing the drug and were subcultured when necessary (20). After 10 weeks (3), cells were exposed to 1 µg/ml of CCH and then maintained in exponentially growing culture (8). The selected cells, named HCA-2/1^{cch}, were grown in RPMI-1640 medium supplemented as above described and with the addition of colchicine (1 µg/ml) to avoid reversion of resistance (17).

Determinations.– P-gly expression was determined by flow cytometry (FACScan, Beckton Dickinson), performing the analysis on cells methanol-fixed (70 %); cells were labelled with JSB-1 monoclonal antibody (Sera-Lab) P-gly (170 kDa) specific (1/25 dilution) and a secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) (1/50 dilution) (24).

Glutathione levels were assayed after monolayer trypsinization. The cells were diluted 1:5 w/v in 5-sulfosalicyclic solution (5 %), and homogenised in a glass Potter-Evelhjeim. The homogenates obtained were centrifuged at 2,000 *g* for 5 minutes at 4 °C. Supernatants were removed and centrifuged in a Beckman microfuge (10,000 *g* for 5 minutes). The

supernatants obtained were used for determination of total glutathione content utilising the DTNB-GSSG reductase recycling assay, described by Anderson (1985) (1).

Glutathione S-transferase activity was measured as described (1985) (25). The assay mixture contained a suitable amount of sample solution (25 µg of protein). The method of Bradford (4) was used for the determination of protein content using bovine serum albumin as protein standard. The activity was expressed as µmol/min/25 µg protein.

Clonogenic assays.– Monolayer clonogenic assays were used to study antineoplastic drug effect on cellular survival. The inhibition in colony formation was taken as drug cytotoxicity measure (15). From exponentially growing culture in drug free medium, 300 cells were seeded per Petri dish and exposed to different doses of drug during 1 hour. They were incubated until colony formation. Colonies were stained with methylene blue (2 %) and methanol (75 %) in distilled water.

Statistical analysis.- The normality of data were tested by the Wilk-Shapiro rankit-plot approximation test, analysing them with the Student's *t*-test and with the one way analysis of variance (ANOVA-I); taking signification levels of 95 % ($p<0.05$) in all cases.

Results and Discussion

P-gly levels were studied during the drug exposure period, harvesting cells on days 0, 26, 53, 74 and 82. Days 0 and 82 correspond to HCA and HCA-2/1^{cch} populations, respectively.

The P-gly fluorescence level increased from channel 33.37 (HCA) to channel 78.43 (HCA-2/1^{cch}). It only increased in the last exposure period (days 74-82). The selected subline showed a 2.35 fold increase in P-gly content, in relation to the parental cell line (Table I). Cellular glutathione content increased gradually during all of the exposure period to colchicine, changing the concentration from 0.39 µM (HCA) (day 0) to 1.25 µM (HCA-2/1^{cch}) (day 82) being the increment of 3.21 fold.

Glutathione S-transferase activity increased from 1.15 µmol/min/25 µg protein (HCA) to 1.46 µmol/min/25 µg protein in the first 4 days of drug exposure (Table 1) and there were no alterations in GST activity from day 4 to 74. On the second exposure period to colchicine (1 µg/ml) and until day 82 (HCA-2/1^{cch}), the GST activity increased up to 2.11 µmol/min/25 µg protein, incrementing 1.84 fold, between the day 0 and 82 (Table I).

The response of HCA and HCA-2/1^{cch} cell lines to colchicine, vinblastine (VBL), vincristine (VCR) and mitomycin C (MMC) was tested. Both cell lines showed cross-resistance to these drugs, obtaining on day 82 (HCA-2/1^{cch}) increments in resistance from 2.62 (VCR) to 5.22 (MMC) fold, in relation to day 0 (HCA) (Table I).

Multidrug resistance phenotype has been observed in cell lines cultured *in vitro* with incrementing doses of cytotoxic agents such as vincristine, colchicine, adriamicine or actinomicine D (22). Resistance profiles varied among different tumours, finding that multiple MDR proteins are expressed in cancer cell lines and may well influence response to chemotherapy (21, 24).

Both cell lines, HCA and HCA-2/1^{cch}, expressed high P-gly levels. These results are similar to those obtained in the mouse

Table I. *P-glycoprotein fluorescence level, glutathione (GSH) content (μM), glutathione S-transferase (GST) activity (μmol/min/25 μg protein) and drug resistance spectrum during the continuous exposure of a human colon adenocarcinoma (HCA) cell line to colchicine.*

Selection day	Fluorescence channel (P-gly)	GSH	GST	Drug resistance (ID_{50}, μg/ml)
0 (HCA)	33.37 ± 2.51	0.39 ± 0.018[c]	1.15 ± 0.034	CCH 0.44; VBL 1.04 VCR 5.98; MMC 0.53
4	n.a.	0.58 ± 0.022[c]	1.46 ± 0.042[d]	
14	n.a.	0.72 ± 0.018[c]	1.46 ± 0.037	
26	34.59 ± 2.92	0.78 ± 0.019[c]	1.44 ± 0.025	
34	n.a.	0.69 ± 0.025[c]	1.43 ± 0.038	
40	n.a.	0.73 ± 0.024[c]	1.44 ± 0.037	
53	39.24 ± 3.05	0.91 ± 0.019[c]	1.54 ± 0.046	
60	n.a.	1.02 ± 0.010[c]	1.56 ± 0.040	
74	29.69 ± 2.79	1.06 ± 0.013[c]	1.51 ± 0.032	
82 (HCA-2/1^{cch})	78.43 ± 5.49[d]	1.25 ± 0.027[c]	2.11 ± 0.067[d]	CCH 1.47[a]; VBL 3.26[a]; VCR 15.68[b]; MMC 2.79[a]

HCA-2/1^{cch}: Human colon adenocarcinoma subline selected with colchicine. Mean ± SD of four replicates. n.a.: Not assayed. CCH: Colchicine. VBL: Vinblastine. VCR: Vincristine. MMC: Mitomycin C. [a] $p<0.001$, [b]$p<0.01$, [d]$p<0.05$ (Student's *t*-test); [c]$p<0.01$ (ANOVA-I).

mammary carcinosarcoma EMT6/CR2.0 cell line (20). These cells are resistant to 2 μg/ml of colchicine and express a 2.5 fold increase in P-gly content respect to the parental line. In this way, high levels of P-gly have been found in the cell lines KB-C2, C-A500 and KCP-4 derived from the human KB-3-1 epidermoid cell line (6). P-gly levels obtained during the selection were similar to CH^{R}B3 MDR Chinese hamster cell line (selected with 3 μg/ml colchicine) and to the CH^{R}C5 cell line, that have been selected with 10 μg/ml of colchicine (16). HCA cells expressed a P-gly level similar to the MDR CEM/VLB5000 subline, which was selected with 5 μg/ml of vinblastine from CCRF-CEM lymphoblastic leukaemia cells (7).

It has been found that increased intracellular GSH or GSH related enzymes have been associated with MDR (2, 23). GSH increment obtained here (3.21) was similar to that reported in ES-2R human ovary carcinoma (11), that expresses a 1.5 increment in GSH level with respect to ES-2 sensitive line. In this way, the increment obtained in GST activity (1.84) was also similar to the activity increment obtained by the same author on ES-2R cells (2.6) (11). In addition, these results suggest that GSH and GST may play an important role in MDR cells that express high levels of P-gly, as in K-H300 leukemic cells (2) or on a panel of colon cancer cell lines selected by repeated mitomycin C exposure (10). In contrast, other authors (18) have selected an alkylating-resistant cell line derived from the MCF-7 human breast carcinoma that do not express the multidrug-resistant phenotype but a significant increase in the cellular content of glutathione has been measured, suggesting that other resistance mechanisms could be operative (18).

HCA and HCA-2/1^{cch} showed cross-resistance to other drugs which were not present in the selection medium. The highest levels usually correspond to the selection agent although, as it has been

reported (17), the cross-resistance to mitomycin C is higher than that observed for colchicine.

The increments obtained in P-gly, GSH levels and GST activity could probably contribute to the MDR phenomenon in this human colon adenocarcinoma cell line. The results obtained suggest that the acquisition of resistance to colchicine produce different alterations in these parameters, suggesting different ways of resistance induction. Identification and quantitative evaluation of drug resistance markers are essential to assess the impact of multidrug resistance (MDR) in clinical oncology. Future studies, using other chemo-selected MDR models, may further help to determine the mechanisms underlying the resistance to anticancer agents.

Acknowledgements

The authors would like to acknowledge Prof. Dr. S. Perán (Biochemistry Department), for providing the colon adenocarcinoma cells. We thank Prof. Dr. J.P. de la Cruz and Mr. A. Pino (Pharmacology Department) and Dr. M. Alvarez (Pathology Department), for their technical assistance.

M. J. RUIZ-GÓMEZ, A. SOUVIRON, M. MARTÍNEZ-MORILLO y L. GIL. *Aumento de glicoproteína-P, glutation y glutation S-transferasa en una línea celular de carcinoma de colon por colchicina.* J. Physiol. Biochem., **56** (4), 307-312, 2000.

La adquisición de resistencia a los agentes anticancerígenos usados en quimioterapia es la principal causa de fallo en el tratamiento del cáncer. El principal mecanismo de resistencia a múltiples drogas (MDR) en células tumorales es la expresión de la glicoproteína-P (P-gly), que actúa como una bomba extrusora de agentes quimioterápicos. Además, se ha observado en células resistentes un incremento en la detoxificación de compuestos mediada por altos niveles de glutation (GSH) y glutation S-transferasa (GST). El objetivo de este trabajo es el estudio de la evolución de los principales marcadores de resistencia a drogas en células tumorales, P-gly, GSH y GST, durante la adquisición de resistencia a colchicina, con el propósito de estudiar el proceso de adaptación y su contribución al fenómeno MDR. Tras exponer una línea celular de adenocarcinoma de colon humano a colchicina durante 82 días, se evalúan durante el tiempo de exposición los niveles de P-gly, GSH y GST por citometría de flujo, espectrofluorimetría y espectrofotometría. Los niveles de P-gly y GSH se incrementan gradualmente durante la exposición hasta 2,35 y 3,21 veces. La actividad GST se incrementa al final del período de exposición (día 82), alcanzando un valor 1,84 veces superior. Además, se obtiene un incremento en la resistencia cruzada a drogas entre 2,62 - 5,22 veces para colchicina, vinblastina, vincristina y mitomicina C. Los aumentos observados de P-gly, GSH y GST podrían contribuir al fenómeno MDR en esta línea celular de adenocarcinoma.

Palabras clave: Citometría de flujo; Glutatión (GSH); Glutatión S-transferasa (GST); Adenocarcinoma de colon humano; Resistencia a múltiples drogas (MDR); Glicoproteína-P.

References

1. Anderson, M. E. (1985): *Meth. Enzymol.,* **113**, 548-555.
2. Benderra, Z., Morjani, H., Trussardi, A. and Manfait, M. (1999): *Adv. Exp. Med. Biol*., **457**, 151-160.
3. Boiocchi, M. and Toffoli, G. (1992): *Eur. J. Cancer.,* **28**, 1099-1105.
4. Bradford, M. M. (1976): *Analyt. Biochem.,* **72**, 248-254.
5. Brian, L. J., Dalton, W., Fisher, G. A. and Sikic, B. I. (1993): *Cancer,* **72**, 3484-3488.
6. Chu, X. Y., Suzuki, H., Ueda, K., Kato, Y., Akiyama, S. and Sugiyama, Y. (1999): *J. Pharmacol. Exp. Ther.,* **288**, 735-741.
7. Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. (1985): *Nature,* **316**, 820-823.
8. Kartner, N., Shales, M., Riordan, J. R. and Ling, V. (1983): *Cancer Res.,* **43**, 4413-4419.
9. Kirschner, L. S., Greenberger, L. M., Hong-Hsu, S. I., Huang Yang, C. P., Cohen, D., Piekarz, R. L., Castillo, G., Han E.-H., Yu, L. and Horwitz, S. B. (1992): *Biochem. Pharmacol.,* **43**, 77-87.
10. Lambert, P. A., Kang, Y., Greaves, B. and Perry, R. R. (1998): *J. Surg. Res*., **80**, 177-181.

11. Lewis, A. D., Duran, G. E., Land, H.-M. and Sikic, B. I. (1992): *Int. J. Radiation Oncology Biol. Phys.,* **22**, 821-824.
12. Lum, B. L., Fisher, G. A., Brophy, N. A., Yahanda, A. M., Adler, K. M., Kaubisch, S., Halsey, J. and Sikic, B. I. (1993): *Cancer,* **72**, 3502-3514.
13. Morales, J. A., Ruiz-Gómez, M. J., Gil-Carmona, L., Souviron, A. and Martínez-Morillo, M. (1995): *Rev. esp. Fisiol. (J. Physiol. Biochem.),* **51**, 43-48.
14. Naito, S., Yokomizo, A. and Koga, H. (1999): *Int. J. Urol*., **6**, 427-439.
15. Pirker, R., Keilhauer, G., Raschack, M., Lechner, C. and Ludwigh, M. (1990): *Int. J. Cancer,* **45**, 916-919.
16. Reeve, J. G., Wright, K. A., Rabbitts, P. H., Twentyman, P. R. and Koch, G. (1989): *J. Natl. Cancer Inst.,* **81**, 1588-1591.
17. Ruiz Gómez, M. J., Gil, L., Souviron, A. and Martínez-Morillo, M. (2000): *J. Physiol. Biochem*., **56**, 33-38.
18. Salvatore, C., Bigioni, M., Iafrate, E. M., Cianfriglia, M. and Manzini, S. (1999): *Anticancer Drugs*, **10**, 663-669.
19. Slovak, M. L., Coccia, M., Meltzer, P. S. and Trent, J. M. (1991): *Anticancer Res.,* **11**, 423-428.
20. Twentyman, P. R., Reeve, J. G., Koch, G. and Wright, K. A. (1990): *Br. J. Cancer.,* **62**, 89-95.
21. Van Brussel, J. P., Van Steenbrugge, G. J., Romijn, J. C., Schröder, F. H. and Mickisch, G. H. (1999): *Eur. J. Cancer*., **35**, 664-671.
22. Van Dam, P. A., Watson, J. V., Lowe, D. G. and Shepherd, J. H. (1992): *Obstet. Gynecol.,* **79**, 616-621.
23. Van der Kolk, D. M., Vellenga, E., Müller, M. and de Vries, E. G. (1999*): Adv. Exp. Med. Biol*., **457**, 187-198.
24. Volm, M., Mattern, J. and Samsel, B. (1992): *Cancer,* **70**, 764-769.
25. Warholm, M., Guthenberg, C., Von Bahr, C. and Mannervik, B. (1985): *Meth. Enzymol.,* **113**, 499-503.