Pyruvate kinase inhibited by L-cysteine as a marker of tumorigenic human urothelial cell lines

M. Gumińska, M. B. Stachurska, B. Christensen[a], V. Tromholt[a], J. Kieler[a], Cz. Radzikowski[b] and D. Duś[b]

Institute of Medical Biochemistry, N. Copernicus Academy of Medicine, Kopernika 7, 31-034 Kraków (Poland), [a] The Fibiger Institute, Danish Cancer Society, Ndr. Frihavnsgade 70, DK-2100 Copenhagen Ø (Denmark), and [b] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, 53-114 Wrocław (Poland)
Received 14 September 1988; accepted 15 March 1989

Summary. It was found that a decrease in electrophoretic mobility of pyruvate kinase (PK) isoenzyme, and an increase of the sensitivity of this enzyme to L-cysteine, were markers of immortalization and tumorigenic properties, respectively, in human urothelial cell lines characterized by different grades of transformation (TGr) in vitro.
Key words. Pyruvate kinase; human urothelial cell lines; L-cysteine; tumor marker.

Our previous metabolic studies[1] demonstrated that the high rate of aerobic glycolysis in Ehrlich ascites tumor cells might be diminished stereospecifically by L-cysteine. The cross-over in the ATP and 2-phosphoenolpyruvate levels pointed to tumor pyruvate kinase (PK) (EC.2.7.1.40) as an enzyme sensitive to L-cysteine. Since this L-cysteine-sensitive PK variant has been found in a broad spectrum of mouse transplanted tumors but not in normal tissues, we accepted it as a cellular marker of neoplastic transformation[2]. Among the three PK isoenzymes found in tumor chromatin extracts, only the slowest was sensitive to L-cysteine[3].

The aim of the present study was to determine whether the L-cysteine-sensitive PK variant might also be used as a marker of tumorigenic properties in vitro in a broad spectrum of human urothelial cell lines.

Urothelial cell lines of normal and transitional cell carcinoma origin[4] can be divided into three groups according to their TGr. The TGr I cell cultures show a very slow growth rate and have a prolonged but finite life-span (4–75 passages). They are fibroblast-independent, non-invasive in vitro and non-tumorigenic in nude mice. The TGr II cultures show an infinite growth potential (> 75 passages) but are also neither tumorigenic nor invasive. Only the TGr III cultures, which also have an infinite growth potential (> 75 passages), produce progressively growing tumors and destroy fragments of the embryonic heart in vitro[5,6]. A more detailed characterization of these cultures has been given by Kieler et al.[5,7] and Christensen et al.[6,8].

Material and methods

Cell lines were established at the Fibiger Institute[4,6], with the exception of HCV 29, obtained from J. Frogh (Memorial Sloan-Kettering Institute, New York) and T 24, obtained from J. Bubenik[9]. Cells were grown in the Fib 41B medium supplemented with 7 non-essential amino acids (Flow Laboratories, Glasgow, Scotland) and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, New York) without antibiotics. For the TGr I cell line tissue culture flasks were coated with collagen IV from the human placenta (Sigma Chemicals Co., St. Louis, USA), as described previously[8]. Cell cultures were grown at 37 °C in an atmosphere enriched with 5% CO_2. Cell monolayers washed twice with PBS were harvested with a rubber policeman. The washed cells were then centrifuged at 800 g for 10 min.

Cell pellets were homogenized in a partly frozen state in a Potter-Elvehjem glass homogenizer in 0.25 M sucrose containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and were centrifuged at 1000 g for 15 min as described by Rickwood[10] to obtain cytosolic and nuclear fractions. Cytosolic fractions after recentrifugation at 100,000 g for 60 min were dialyzed overnight at 4 °C against 20 mM Tris-HCl buffer pH 7.4, containing 115 mM KCl, 10 mM $MgCl_2$ and 2 mM EDTA, and were used for enzymatic determinations.

Chromatin was obtained from the nuclear fraction according to Bloom and Anderson[11], as described previously[3]. The chromatin pellets were rehomogenized in 75 mM Tris-HCl buffer, pH 7.4, containing 250 mM KCl, 25 mM $MgCl_2$, 2 mM EDTA, and were centrifuged at 6000 g for 10 min. The chromatin extracts were used for PK determinations.

The PK activity was determined spectrophotometrically at 340 nm according to Bücher and Pfleiderer[12], as described previously[13], under conditions of pseudo-zero-order kinetics. 2-Phosphoenolpyruvate (x 2Na, x $7H_2O$) from Calbiochem (Switzerland), ADP, NADH and LDH from Boehringer (Mannheim, FRG) and L-cysteine from Serva Feinbiochemica (Heidelberg, FRG) were used. Protein was determined according to Lowry et al.[14].

Disc electrophoresis, according to Davis[15], was performed in 7.5% polyacrylamide gel as described previously[3]. Each tube contained 30–60 µg of protein. For enzymatic studies the gel from each tube of 5 mm in diameter was cut up into slices 1.5 mm thick. Each slice was transferred to a separate test tube, extracted for 24 h at 4 °C with 0.2 ml of the buffer for PK determination (75 mM Tris-HCl buffer, 100 mM KCl, 25 mM $MgCl_2$, 2 mM EDTA, pH 7.4).

Results and discussion

The tumor growth rate is positively correlated with the overall rate of aerobic glycolysis and PK activity[16,17], since PK, as one of the key glycolytic enzymes[18], is di-

rectly involved in ATP formation in both cytosolic and nuclear compartments. The total cytosolic PK activity was generally higher in TGr III human urothelial cell cultures than in TGr II or TGr I (table 1). These results support the concept of an altered enzymatic strategy in rapidly growing cells[16,17], as was found in rat hepatomas[19]. The nuclear PK activities were lower than the cytosolic ones and they were of similar magnitude in all the cell lines studied (table 1).

Both the cytosolic and the nuclear PK showed a similar pattern of sensitivity to L-cysteine (table 1). The L-cysteine-sensitive PK variant appeared only in the TGr III cell lines. The maximum inhibition of PK at the given L-cysteine concentration, or at higher ones, was always stabilized at about 50% of the control activity. This indicated that in addition to the L-cysteine-sensitive variant of PK, the TGr III cell lines also contain L-cysteine-insensitive PK fractions.

Usually the amount of tissue culture material is not sufficient for PK purification. However, in polyacrylamide gel electrophoresis the tissue culture chromatin extracts showed an equivalent numbers of PK isoenzymes and protein bands, which stained with Amido Black 10 B[3]. This indicated that the extracts were suitable for further comparative studies of PK.

In all the chromatin extracts three PK isoenzymes (alpha, beta, gamma) were found in polyacrylamide gel electrophoresis (fig. 1). As in the liver[3], the mobility of isoenzymes in the TGr I culture was greater than in the TGr II or TGr III one. Only the slow-migrating gamma isoenzyme of TGr III cells was sensitive to L-cysteine. A similar result has recently been found in the Ehrlich ascites tumor and in Morris hepatoma 7777[3].

Although the commonly-known PK types are related to their tissue origin, i.e. the liver (L), the muscles (M) and the kidneys (K or M_2), they are also present in many fetal and adult tissues including tumors[20]; therefore the names alpha, beta, gamma (used in our paper in the sequence of their decreasing electrophoretic mobility) seem to be better suited to various cultured cells. Imamura and Tanaka[21] found that at pH 8.2 the L-type moves fastest in polyacrylamide gel, the M-type has an intermediate mobility and the M_2 one moves slowest towards the anode. Our gamma isoenzyme corresponds to the M_2 type, but the difference found between $gamma_1$ (faster) from the TGr I culture and $gamma_2$ (slower) from the TGr II and III cells is in accordance with the observation that tumor PK isoenzymes with the highest pI are more cathodic than M_2 (K) isoenzymes[20].

The main difference between the slow-migrating $gamma_2$ isoenzyme of PK from the TGr II and TGr III cells is the presence of sensitivity to L-cysteine in the TGr III isoenzyme (fig. 1, table 2). Table 2 demonstrates a dose-dependent inhibition, which at a given L-cysteine concentration of 0.1 mM is more pronounced in the gamma isoenzyme than in the whole cytosolic fraction or chromatin extracts, but at higher L-cysteine concentrations even approached zero.

Table 1. Pyruvate kinase (PK) activity in cytosolic fractions and chromatin extracts of human urothelial cell lines (TGr I–III), and the effects of L-cysteine (as % of control activity without L-cysteine)

TGr	Cell line	Cytosolic fractions PK (mU/mg protein)			Effect of L-cysteine percent of control PK		Chromatin extracts PK (mU/mg protein)			Effect of L-cysteine percent of control PK	
		X	±SD	(n)	0.05 mM	0.1 mM	X	±SD	(n)	0.05 mM	0.1 mM
TGr I	Hu 1125	980	–	(2)	100	100	829	–	(2)	100	100
TGr II	Hu 609	932	159	(3)	100	100 100	861	149	(3)	100	100 100
	Hu 1734	1246	349	(3)	100	100 100	923	239	(3)	100	100 100
	HCV 29	1513	269	(4)	100 100	100 100	1124	143	(4)	100 100	100 100
TGr III	Hu 609 T_{LLH}	5729	1130	(3)	62	45 57	1070	182	(3)	90	70 62
	HU 609 T_{MV}	4765	1045	(3)	78	65 55	718	187	(3)	81	62 47
	HCV 29 T	3839	985	(4)	79 76	61 43	1428	478	(4)	83 87	68 55
	Hu 456	6084	2115	(5)	61 57	39 43 42	1357	184	(4)	73	44 48 55
	Hu 549	5666	784	(3)	61	54 49	959	75	(3)	80	43 51
	Hu 961 a	5280	–	(2)	73	54	1717	–	(2)	85	71
	Hu 1703 He	5647	781	(4)	56 60	39 38	1202	289	(4)	87	67 58
	T 24	3238	135	(4)	72 48	46 36	1400	356	(3)	87	69 50

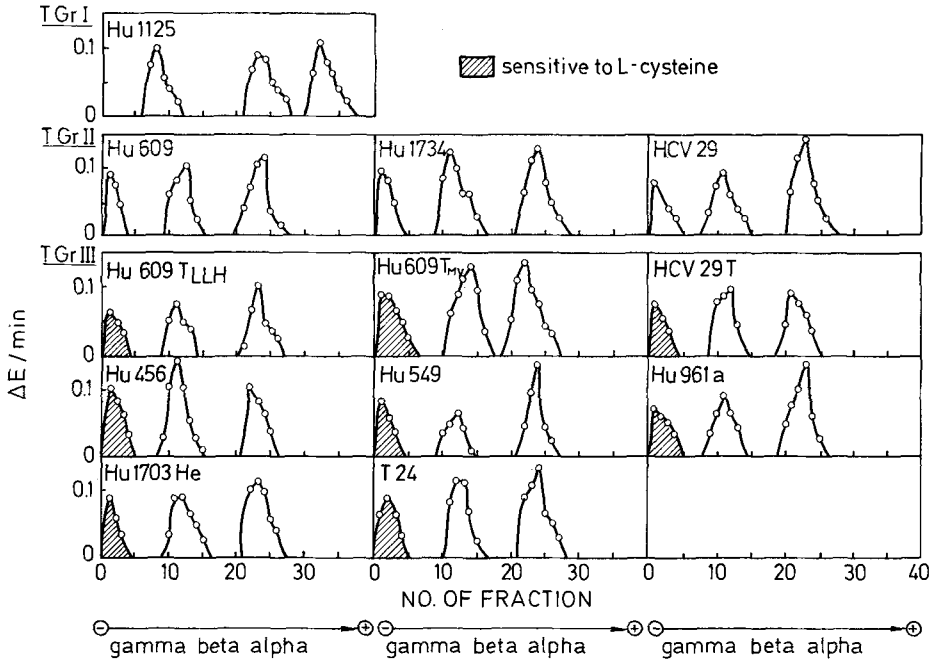

Figure 1. An isoenzyme pattern of pyruvate kinase from chromatin extracts of various urothelial cell lines (TGr I–III) after polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8.4. Plots of ΔE/min vs No. of fractions. Hatched areas: isoenzymes sensitive to L-cysteine.

Two sublines were isolated from the T 24 cell line: a non-tumorigenic one (T 24a) and a highly tumorigenic (T 24b) one (Tromholt, in preparation). The non-tumorigenic T 24a subline, which contains four PK isoenzymes, (as in the combination of the TGr I and TGr II isoenzymes) has lost its sensitivity to L-cysteine (fig. 2). Further studies will show whether the T 24 cell line is heterogeneous, or whether some features of the TGr III cell are reversible.

Although the regulatory role of various amino acids in relation to PK is well known[20], no L-cysteine-sensitive PK variant has been described as yet. The metabolic role of the new PK isoenzyme in tumors is obscure. According to our previous studies, it may influence cell

Table 2. Effect of L-cysteine on pyruvate kinase isoenzymes from chromatin extracts of various urothelial cell lines (TGr I–III). The results are expressed as percent of control activity without L-cysteine.

TGr	Cell line	Effect of L-cysteine (mM) on isoenzymes					
		alpha		beta		gamma	
		0.05	0.10	0.05	0.10	0.05	0.10
TGr I	Hu 1125	100	100	100	100	100	100
TGr II	Hu 609	100	100	100	100	100	100
		100	100	100	100	100	100
	Hu 1734	100	100	100	100	100	100
		100	100	100	100	100	100
	HCV 29	100	100	100	100	100	100
		100	100	100	100	100	100
TGr III	Hu 609 T_{LLH}	100	100	100	100	53	29
	609 T_{MV}	100	100	100	100	44	28
		100	100	100	100	61	35
	HCV 29 T	100	100	100	100	47	24
		100	100	100	100	62	33
	Hu 456	100	100	100	100	58	42
		100	100	100	100	46	28
	Hu 549	100	100	100	100	77	69
		100	100	100	100	58	45
	Hu 961 a	100	100	100	100	59	29
	Hu 1703 He	100	100	100	100	61	43
		100	100	100	100	46	26
	T 24	100	100	100	100	60	33
		100	100	100	100	48	20

For details see legends to fig. 1 and table 1.

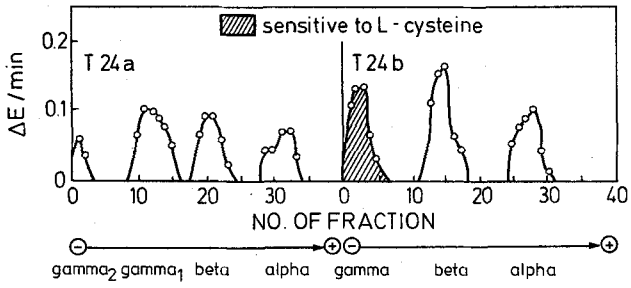

Figure 2. A comparison of pyruvate kinase isoenzymes from chromatin extracts of T24 two sublines:the non-tumorigenic T 24a and the tumorigenic T 24b. Hatched area: isoenzyme sensitive to L-cysteine.

metabolism[1,2]. The sensitivity to L-cysteine also points to changes in the primary structure of PK, and thus in the cell genotype, which take place during a multistage process of carcinogenesis.

The altered sensitivity of tumor PK to normal signal molecules, such as ATP[3,22] or fatty acids[23], also brings about several metabolic consequences[24].

It can be concluded that the decrease in electrophoretic mobility of the slow-migrating PK gamma isoenzyme and the appearance of its sensitivity to L-cysteine could be used as cellular markers of immortalization and tumorigenic transformation, respectively, in human urothelial cell lines. It seems that the two coupled features, tumorigenicity in nude mice and sensitivity to L-cysteine, might be useful in monitoring successive stages of carcinogenesis in vitro.

Since numerous tumor markers present in the body fluids can be used for laboratory diagnostics of neoplastic diseases in living organisms[25], a new attempt has been made to evaluate the described PK isoenzyme sensitive to L-cysteine for this purpose as well, using in vivo studies.

Acknowledgments. The authors would like to thank Ms Ellen Møllgaard for her excellent technical assistance. The support of the Neye Foundation is gratefully acknowledged.

1 Kędryna, T., Gumińska, M., and Marchut, E., Biochim. biophys. Acta *764* (1983) 64.
2 Marchut, E., Gumińska, M., Kędryna, T., Radzikowski, C., and Kuśnierczyk, H., Experientia *44* (1988) 25.
3 Gumińska, M., Stachurska, M. B., and Ignacak, J., Biochim. biophys. Acta *966* (1988) 207.
4 Vilien, M., Christensen, B., Wolf, H., Rasmussen, F., Hou-Jensen, C., and Povlson, C. O., Eur. J. Cancer clin. Onc. *19* (1983) 775.
5 Kieler, J., Cancer Metastasis Rev. *3* (1984) 265.
6 Christensen, B., Kieler, J., Vilien, M., Don, P., Wang, C. Y., and Wolf, H., Anticancer Res. *4* (1984) 319.
7 Kieler, J., Christensen, B., Ottesen, S., Tromholt, V., and Ostrowski, K., Anticancer Res. *7* (1987) 959.
8 Christensen, B., Kieler, J., and Bem, W., Anticancer Res. (1989) in press.
9 Bubenik, J., Baresova, M., Vicklicky, V., Jakoubkova, J., Sainorova, H., and Donner, J., Int. J. Cancer *11* (1973) 765.
10 Rickwood, D., and Birnie, G. D., in: Subnuclear Components. Preparation and Fractionation, p. 129. Ed. G. D. Birnie. Butterworths Ltd., London 1976.
11 Bloom, K. S., and Anderson, J. N., J. biol. Chem. *253* (1978) 4446.
12 Bücher, T., and Pfleiderer, G., in: Methods of Enzymology, vol. 1, p. 435. Eds S. P. Colowick and N. O. Kaplan. Academic Press, New York 1955.
13 Gumińska, M., Ptak, W., and Zembala, M., Enzyme *19* (1975) 24.
14 Lowry, O. H., Rosebrough, W. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
15 Davis, B. J., Ann. N.Y. Acad. Sci. *121* (1964) 404.
16 Weber, G. N., Engl. J. Med. *296* (1977) 486.
17 Weber, G., Naturwissenschaften *55* (1968) 418.
18 Weber, G., in: Advances in Enzyme Regulation, vol. 7, p. 15. Ed. G. Weber. Pergamon Press, Oxford 1969.
19 Criss, W. E., Biochem. biophys. Res. Commun. *35* (1969) 901.
20 Ibsen, K. H., Cancer Res. *37* (1977) 341.
21 Imamura, K., and Tanaka, T., J. Biochem. *71* (1972) 1043.
22 Gosalvez, M., García-Suarez, S., and López-Alacron, L., Cancer Res. *38* (1978) 142.
23 Marchut, E., Gumińska, M., and Kędryna, T., Acta biochem. Pol. *44* (1986) 6.
24 Gosalvez, M., Perez-García, J., and Weinhouse, S., Eur. J. Biochem. *46* (1974) 133.
25 Ziegenbein, R., in: Tumor-Marker, Fortschritte der Onkologie, vol. 8. Akademie-Verlag, Berlin 1982.

0014-4754/89/060571-04$1.50 + 0.20/0

Synthesis of $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2, Hyp^5] enkephalin amide, a new highly potent analgesic enkephalin-related glycosyl peptide

J. L. Torres*, I. Haro, G. Valencia, F. Reig and J. M. García-Antón

Peptide Research Section, Centre d'Investigació i Desenvolupament, C.S.I.C., C/Jordi Girona 18–26, E-08034 Barcelona (Spain)

Received 27 May 1988; accepted 17 February 1989

Summary. A new series of O-glycosyl enkephalins has been prepared, following a convergent strategy, with high chemical yields. The galactosyl analogue, $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2, Hyp^5] enkephalin amide proved to be one of the most potent in vivo opioid agonists synthesized up to now.

Key words. Analgesic peptides; enkephalin analogues; galactosylpeptides; glycosylpeptides; peptide synthesis.

The search for more potent and selective biologically active analogues is one of the main goals in peptide chemistry. This aim has been pursued in our laboratory by producing different modifications of the enkephalins[1,2], two pentapeptides originally discovered by Hughes et al.[3]. The promising results obtained by