Biochem. J. (1985) **229**, 711–715
Printed in Great Britain

Tumourigenicity, cell-surface glycoprotein changes and ornithine decarboxylase gene pattern in Ehrlich ascites-carcinoma cells

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(*Received 25 February 1985/13 May 1985; accepted 16 May 1985*)

We selected a 2-difluoromethylornithine-resistant Ehrlich ascites-carcinoma cell line that grows in the presence of 20mM-difluoromethylornithine. These cells contain 10–20 times the normal amount of hybridizable sequences for ornithine decarboxylase (EC 4.1.1.17) in their genomic DNA. We used these gene-amplified cells, their revertant counterparts (grown in the absence of the drug after an established gene amplification) and tumour cells grown in the presence of putrescine to investigate the changes of ornithine decarboxylase gene pattern and simultaneously occurring phenotypic changes, such as tumourigenicity and the expression of cell-surface glycoproteins. In the tumour cells reverted back to the normal gene frequency, not only did the amplified sequences disappear, but there were also signs of gene re-arrangements seen as a 'gene jump', when a signal evidently moved to a heavier restriction fragment. Similar gene re-arrangement likewise occurred in cells exposed to putrescine. Although the wild-type tumour cells and the gene-amplified cells readily grew in the peritoneal cavity of mice, the revertant cells and the putrescine-treated cells had lost their tumourigenicity in mice. Gene-amplified tumour cells and the revertant cells showed distinct changes in their surface glycoprotein pattern in comparison with the parental cell line. These findings indicate that alterations of ornithine decarboxylase gene pattern/dosage may be associated with phenotypic changes possibly related to the tumourigenicity of these carcinoma cells.

ODC, the rate-controlling enzyme of polyamine biosynthesis, is one of those few enzymes the genes of which easily undergo amplification under appropriate selection pressure (Schimke, 1984). As documented by gene determination, it is obvious that several mouse cell lines amplify their ODC genes either through spontaneous mutations or when exposed for longer periods of time to DFMO (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985), a mechanism-based irreversible inhibitor of the enzyme (Metcalf *et al.*, 1978).

With stepwise increments of the concentration of DFMO, we previously selected cultured Ehrlich ascites-carcinoma cell lines resistant to DFMO concentrations up to 50mM (Alhonen-Hongisto *et al.*, 1985). The over-production of ODC in these cells was based on greatly enhanced expression of the message and a 10–20-fold increase in the DNA sequences coding for this enzyme (Alhonen-Hongisto *et al.*, 1985).

Although, once it had occurred, the gene amplification appeared to be relatively stable, the tumour cells reverted towards the normal gene frequency in the absence of the inhibitor pressure over a period of several months (Alhonen-Hongisto *et al.*, 1985).

We have now inoculated mice with the gene-amplified Ehrlich ascites-carcinoma cells, with tumour cells reverted back from the amplified state to the original gene dosage, and with cells exposed to putrescine, in order to elucidate whether different ODC gene dosage is associated with changes of the growth characteristics of the carcinoma cells *in vivo*. Our results indicate that the different selection protocols used resulted in the establishment of cell lines with altered tumourigenicity possessing a typically altered ODC gene pattern.

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, 2-difluoromethylornithine; kb, kilobases.

Experimental

Cell cultures

Ehrlich ascites-carcinoma cells were grown in suspension cultures in RPMI 1640 medium supplemented with 5% (v/v) pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland) and antibiotics. The selection of DFMO-resistant tumour cells was achieved by stepwise increments of the concentration of DFMO as described by Alhonen-Hongisto *et al.* (1985).

Animals

Female NMRI mice were used in the inoculation experiments. The cells were harvested from the peritoneal cavity of the mice with the aid of 0.9% NaCl, subjected to hypo-osmotic treatment to remove contaminating red cells and weighed or used for further analyses.

Materials

DFMO was generously given by Centre de Recherche Merrell International (Strasbourg, France). [^{32}P]dCTP (sp. radioactivity 410Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.).

Analytical methods

DNA was extracted by the method of Blin & Stafford (1976). Isolated DNA was digested with *Eco*RI or *Bam*HI restriction endonuclease and electrophoresed in 0.9% agarose gels. The fragments were transferred on to nitrocellulose filters (Southern, 1975), hybridized to a nick-translated (Rigby *et al.*, 1977) (sp. radioactivity 10^8 c.p.m./μg) plasmid pODC16, which included the ODC cDNA sequence (Kontula *et al.*, 1984; Jänne *et al.*, 1984), and autoradiographed. For surface-glycoprotein analysis, the tumour cells were labelled by the periodate/NaB^{3}H$_4$ technique (Gahmberg & Andersson, 1977) and run on 8% polyacrylamide gels (Laemmli, 1970). Fluorography was performed as described by Bonner & Laskey (1974).

Results

The restriction analysis in Fig. 1 shows that the development of DFMO-resistance was associated with an amplification of ODC sequences residing mainly in an *Eco*RI fragment of about 6.5kb (Fig. 1, lane 2). In comparison with the wild-type cells (lane 1), it is possible that the amplification involved also other restriction fragments (lane 2). Lanes 3 and 4 (Fig. 1) represent restriction analyses of genomic DNA obtained from gene-amplified tumour cells transferred into drug-free medium 6 (lane 3) or 2 (lane 4) months before analysis. In addition to the disappearance of the amplified sequences in the 6.5kb fragment, the revertant cells displayed another interesting change in their ODC gene pattern. As shown in Fig. 1, the ODC gene pattern in the wild-type Ehrlich ascites cells (lane 1) included two closely spaced *Eco*RI fragments of about 4 kb size (lanes 1 and 2, arrows). These fragments were retained with no apparent change in intensity during the amplification process (lane 2). However, on the return to normal gene frequency in the absence of the selection pressure, one of these fragments almost disappeared and evidently moved to a heavier restriction fragment (lanes 3 and 4, arrows). A similar change was also evident in *Bam*HI digests (results not shown).

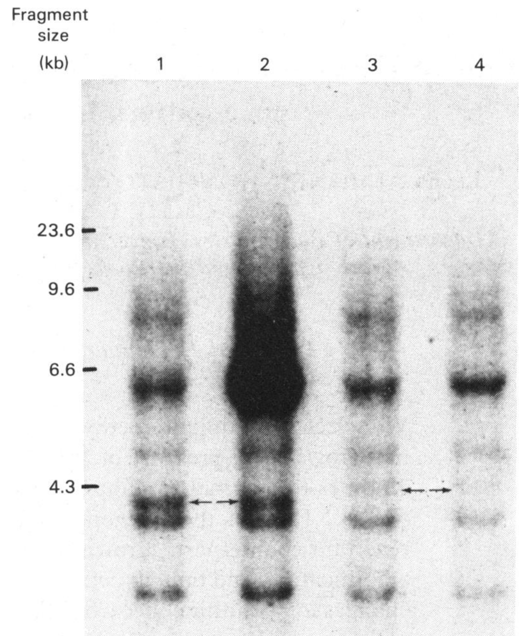

Fig. 1. *Restriction analysis (EcoRI) of ODC sequences in genomic DNA isolated from Ehrlich ascites-carcinoma cells*
DNA was isolated from untreated tumour cells (lane 1), from tumour cells that were grown in the continuous presence of 20mM-DFMO for about 12 months (lane 2), from cells that were grown in the presence of 20mM-DFMO until gene amplification was established, and then transferred into drug-free medium, 6 (lane 3) or 2 (lane 4) months before the analysis. The size markers are shown to the left.

The tumourigenic properties of wild-type tumour cells, gene-amplified cells and revertant cells (once amplified and then reverted back to the normal gene frequency in the absence of selection pressure) are presented in Table 1. Although there was no difference between the tumour masses (on day 12 after the inoculation) obtained from mice inoculated with normal tumour cells or with gene-

Table 1. *Tumourigenicity of Ehrlich ascites-carcinoma cells with different ODC gene patterns*
Female NMRI mice (six in each group) were inoculated with the appropriate tumour cells (7×10^6 cells) or served as controls for spleen weight. The animals were killed 12 days after the inoculation. The invasiveness is defined as the attachment of the tumour cells to the peritoneal organs. Results are means ± S.D.: $*P<0.05$ and $**P<0.01$ as compared with the animals not inoculated.

Tumour cells used for inoculation	No. of deaths	No. of animals with tumour	Tumour cell mass (g)	Spleen wt. (mg)	Invasiveness
None	0/6	0/6	—	179 ± 35	−
Untreated	0/6	6/6	2.49 ± 0.31	221 ± 59	−
Gene-amplified	1/6	6/6	2.26 ± 0.56	272 ± 65*	+
Revertant	0/6	0/6	—	318 ± 79**	−

amplified cells, the latter cells were clearly more invasive, as they tended to spread all over the peritoneum, making the harvesting of the cells difficult. The gene-amplified cells also grew with little ascitic fluid, as a very dense suspension. The spleen weights of mice inoculated with these cells were higher than those of uninoculated mice or mice inoculated with the wild-type cells (based on three different experiments) (Table 1). Although the revertant cells grew most rapidly in the culture (the doubling time was half of that of the untreated cells or the gene-amplified cells), these cells appeared to have lost their tumourigenicity in the mouse. None of the animals inoculated with the revertant cells showed any signs of ascites tumour 12 days after the inoculation (Table 1) (at that time the mice inoculated with the wild-type tumour cells, and especially those inoculated with the gene-amplified cells, were dying from the tumour burden). The only change was a grossly elevated spleen weight.

We also exposed tumour cells to putrescine, the product of ODC, in order to study whether the inhibition of the enzyme by putrescine influences the ODC-gene amplification. The Southern-blot analyses depicted in Fig. 2 are from an experiment where Ehrlich ascites-carcinoma cells were exposed to 1 mM-putrescine for 2 months. Although it is difficult to say whether the total gene dosage was changed, it is obvious that the exposure to putrescine (Fig. 2, lanes 2 and 3) resulted in a similar change to that seen in the revertant cells (Fig. 2, lanes 1 and 4, arrows). In addition, the putrescine-exposed cells contained ODC activity that was only a fraction of that found in the wild-type cells (results not shown).

Interestingly, just like the revertant cells (Table 1), the putrescine-treated cells with the re-arranged gene pattern for ODC also appeared to have lost the ability to proliferate in their normal host animal, as shown in Table 2. In this particular experiment (Table 2) the untreated cells (with normal gene pattern) killed the mice more rapidly

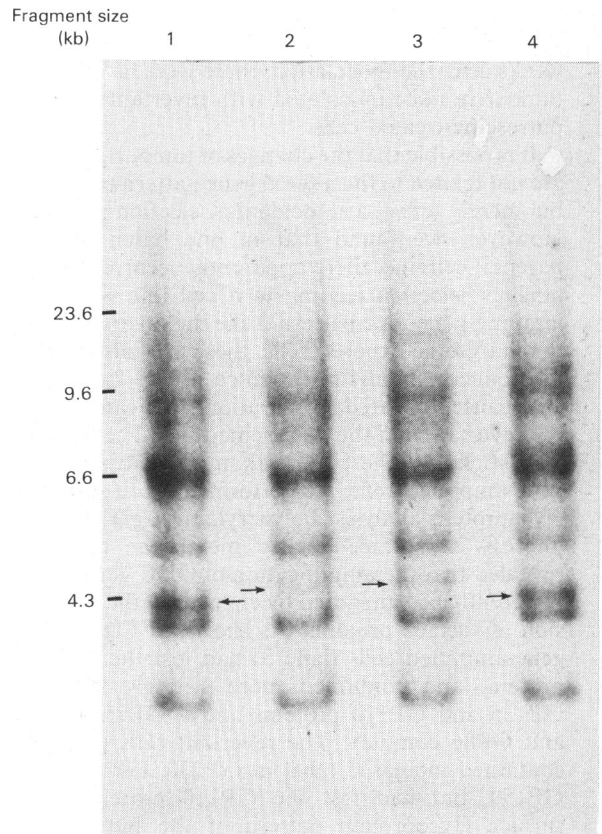

Fig. 2. *Restriction analysis of ODC sequences in genomic DNA of carcinoma cells grown in the absence or presence of putrescine*
DNA was isolated from untreated Ehrlich ascites-carcinoma cells (lanes 1 and 4) or from cells grown in the presence of 1 mM-putrescine for about 2 months (lanes 2 and 3). The size markers are shown to the left.

than did the gene-amplified cells; however, the cause of death by the parental cells appeared to be based not on tumour mass but on excessive production of ascitic fluid and mechanical induc-

Table 2. *Tumourigenicity of Ehrlich ascites-carcinoma cells with re-arrangements in ODC gene pattern*

Female NMRI mice (six in each group) were inoculated with the appropriate tumour cells (8×10^6 cells). The animals were examined 23 days after the inoculation or when they died.

Tumour cells used for inoculation	No. of deaths	No. of animals with tumour
Untreated (normal gene pattern)	6/6	6/6
Untreated (jumped gene)	0/6	0/6
Gene-amplified	3/6	6/6
Revertant	0/6	0/6
Putrescine-exposed	0/6	0/6

tion of haemorrhages in the peritoneum. At 3 weeks after the inoculation, there were no signs of tumour in mice inoculated with revertant cells or putrescine-treated cells.

It is possible that the changes of tumourigenicity are not related to the altered gene pattern of ODC, but merely reflect a coincidental selection process. However, we found that in one batch of the parental cell line, there apparently occurred spontaneous selection leading to a cell line with the jumping-gene-type pattern. Like the revertant and the putrescine-exposed cells, these cells also failed to produce tumours in the mice (Table 2).

Because repeated observations indicated that the revertant and the putrescine-treated cells were less 'sticky' in the culture than the untreated or gene-amplified cells, we performed a cell-surface-glycoprotein analysis. Polyacrylamide-gel electrophoresis of surface-labelled membrane proteins revealed that the amplification of ODC genes was apparently accompanied by changes in the expression of surface proteins. As shown in Fig. 3, the gene-amplified cells (lane 3) had lost the GP230 protein, and contained more strongly labelled GP135 and GP110 proteins and a GP95, GP92 and GP86 complex. The revertant cells (lane 4) contained increased label in GP230, GP180 and GP150, but had lost the GP110 protein. The surface glycoprotein pattern of the putrescine-treated cells was just like that found in the revertant cells (results not shown).

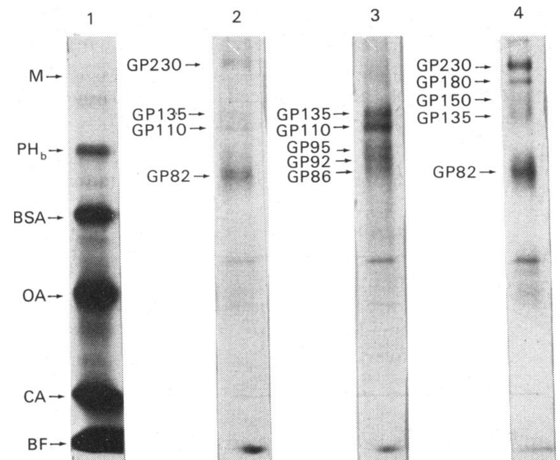

Fig. 3. *Polyacrylamide-gel electrophoresis of* ^{14}C*-labelled standard proteins* (*lane 1*), *of membrane proteins from untreated Ehrlich ascites-carcinoma cells* (*lane 2*), *from cells bearing amplified genes for ornithine decarboxylase* (*lane 3*) *and from cells reverted back to the normal gene frequency* (*lane 4*) *after growth for 6 months in the absence of DFMO*

The M_r markers were as follows: M, myosin; PH$_b$, phosphorylase *b*; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; BF, buffer front. The cells were labelled by the periodate/NaB^3H_4 technique and run on 8%-acrylamide gels. The apparent M_r values of the labelled glycoproteins are indicated: GP230, surface glycoprotein with an apparent M_r of 230000 etc.

Discussion

Although it is well established that the amplification of mammalian genes often involves a much larger DNA segment than the target gene (Schimke, 1984; Stark & Wahl, 1984), the nature of the co-amplified sequences is poorly understood. One of the few defined examples is the co-amplification of ribosomal genes in cells exposed to *N*-(phosphonacetyl)-L-aspartate (Stark & Wahl, 1984) with the genes of the trifunctional enzyme (CAD) required for pyrimidine synthesis. Another example is the expression of several amplified genes in Chinese-hamster fibroblasts overproducing adenylate deaminase (Debatisse *et al.*, 1984).

It is apparent that the amplification and de-amplification cycles of ODC genes are associated with gene re-arrangements. On return to the normal gene frequency from the amplified state, not only did the tumour cells lose the amplified sequences (mainly residing in the 6.5kb *Eco*RI restriction fragment), but the pair of fragments of about 4kb, typical of the parental and gene-amplified cells, was distinctly and consistently (in different revertant cell lines) modified to give rise to a 'gene jump'. A similar change was also induced by exposure to putrescine. At first sight it is difficult to understand what is common to these two situations, i.e. removal of the selection pressure (DFMO) from the gene-amplified cells and the exposure of the parental tumour cells to putrescine. Both cases, however, are characterized by the fact that there no longer was a need for high ODC activity, as the inhibitor was removed or the cells were supplied with the product of ODC. Although the simultaneous changes in tumourigen-

icity may just be a coincidence resulting from the inherent genetic lability of the tumour cells, these changes could also be related to the altered gene pattern (the jumping gene) of ODC. In addition to the two different selection protocols used, removal of the inhibitor from the amplified cells or exposing the parental cells to putrescine, we obtained evidence that the gene jump can also occur spontaneously without any selection pressure. In each of these three cases, the apparent rearrangement of ODC genes was accompanied by a distinct loss of tumourigenicity.

The differences in the expression of surface glycoproteins between the different cell lines (parental, gene-amplified, revertant and putrescine-exposed) may also be a coincidence and not related to the changes of ODC gene pattern. In fact, we found, using polyacrylamide-gel electrophoresis, that the expression of several cytosolic proteins (besides ODC) was enhanced in the gene-amplified cells in comparison with the wild-type cells and that some of the proteins present in the parental cells were no longer found in the gene-amplified cells (L. Alhonen-Hongisto, P. Nikula, R. Sinervirta & J. Jänne, unpublished work).

The view that tumourigenicity and the expression of surface-located glycoproteins may be coordinately regulated is strengthened by a finding (Collard *et al.*, 1985) indicating that transfection of 3T3 cells with human oncogenes induced tumourigenicity associated with altered expression of surface glycoproteins. Similarly, revertants which had lost the oncogenes also lost the tumourigenic potential and the transfection-related glycoproteins (Collard *et al.*, 1985).

It is of course much too soon to draw any conclusions regarding a cause-and-effect relationship between the changes in ODC gene dosage and the phenotypic changes observed. The answers to this and other questions can come only after we have a much better perspective about the mechanisms of gene amplification in general.

The skilful technical assistance of Ms. Raija Laine and Ms. Ulla Katajarinne and the secretarial work of Ms. Heini Howard are gratefully acknowledged. This investigation was supported by a Research Grant from the University of Helsinki, by the Finnish National Research Council for Natural Sciences, by the Sigrid Juselius Foundation, by the Finnish Cultural Foundation and by N.I.H. grants HD-13541 and 2 ROI CA26294-OH.

References

Alhonen-Hongisto, L., Kallio, A., Sinervirta, R., Seppänen, P., Kontula, K. K., Jänne, O. A. & Jänne, J. (1985) *Biochem. Biophys. Res. Commun.* **126**, 734–740

Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3**, 2303–2308

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88

Collard, J. G., van Beck, W. P., Janssen, J. W. G. & Schijven, J. F. (1985) *Int. J. Cancer* **35**, 207–214

Debatisse, M., de Saint Vincent, B. R. & Buttin, G. (1984) *EMBO J.* **3**, 3123–3127

Gahmberg, C. G. & Andersson, L. C. J. (1977) *J. Biol. Chem.* **252**, 5888–5894

Jänne, O. A., Kontula, K. K., Isomaa, V. V. & Bardin, C. W. (1984) *Ann. N.Y. Acad. Sci.* **438**, 72–84

Kahana, C. & Nathans, D. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3645–3649

Kontula, K. K., Torkkeli, T. K., Bardin, C. W. & Jänne, O. A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 731–735

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

McConlogue, L., Gupta, M., Wu, L. & Coffino, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 540–544

Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, J. & Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553

Rigby, P. W. J., Dieckman, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251

Schimke, R. T. (1984) *Cell* **37**, 705–713

Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517

Stark, G. R. & Wahl, G. M. (1984) *Annu. Rev. Biochem.* **53**, 447–491