Inhibition of glutaminase expression by antisense mRNA decreases growth and tumourigenicity of tumour cells

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Phosphate-activated glutaminase has a critical role in tumours and rapidly dividing cells and its activity is correlated with malignancy. Ehrlich ascites tumour cells transfected with the pcDNA3 vector containing an antisense segment (0.28 kb) of rat kidney glutaminase showed impairment in the growth rate and plating efficiency, as well as a shortage in the glutaminase protein and activity. The C-terminal segment used is well conserved in all glutaminase sequences known. The transfected cells, named 0.28AS-2, displayed remarkable changes in their morphology compared with the parental cell line. The 0.28AS-2 cells also lost their tumourigenic capacity *in vivo*. Control mice developed an

ascitic tumour, with a lifespan of 16 ± 1 days, when inoculated with 10^7 cells/mouse; on the contrary, animals inoculated with transfected cells up to 2.5 times the cell numbers of control mice did not develop tumours and behaved as healthy animals. The ability to revert the transformed phenotype of antisense-transfected cells confirms the relevance of glutaminase in the transformation process and could provide new ways for the study of gene therapy.

Key words: cancer, cell proliferation, gene expression blockage, glutamine therapy.

INTRODUCTION

Tumour cells need a constant supply of both energy and nitrogen substrates. Glutamine, the most abundant amino acid in the plasma, is described as the main physiological nitrogen vehicle between different mammalian tissues [1]. The presence of a tumour produces great changes in the host glutamine metabolism a few days after tumour transplantation, resulting in an increase in the circulating glutamine [2]. Before being metabolized, glutamine must be transported across both the plasma membrane [3] and the inner mitochondrial membrane [4], since phosphate-activated glutaminase, the first step in the glutaminolytic process, is loosely anchored to the inner leaflet (on the matrix side) of the mitochondria [5]. Malignant cells transport glutamine at a faster rate than non-malignant cells across their plasmatic membranes by means of Na^+ -dependent A and ASC amino acid-transport systems [3], and across the inner mitochondrial membrane by a carrier-mediated system [4].

Many experimental data provide evidence that phosphate-activated glutaminase (EC 3.5.1.2) is correlated with malignancy in tumours [6,7]. The enzyme is differentially expressed; its activity reaches a maximum during exponential growth phase, and the relative abundance of glutaminase mRNA significantly increases as compared with the stationary growth phase [8,9]. Thus glutaminase activity parallels the levels of poly(A)⁺ RNAs encoding for mitochondrial glutaminase. The enzyme has been isolated to homogeneity from Ehrlich ascites carcinoma; the tumour enzyme shows kinetic behaviour that resembles kidney-type glutaminase [10,11].

The presence of high levels of glutaminase seems to be a common feature of rapidly dividing cells. Nevertheless, neither energy nor biosynthetic requirements can totally explain the very high glutaminolytic flux found in tumours and rapidly dividing cells (see [12,13] for reviews). Crabtree and Newsholme [14] suggest that active glutaminolysis is needed for preparing cells to

supply energy or metabolic precursors when required, and consequently cells behave as dissipative systems. In spite of the relevance of glutaminase in cancer growth, studies on this enzyme are scarce.

The present study was undertaken to gain direct evidence on the role of glutaminase in tumour growth and proliferation. Ehrlich ascites carcinoma cells cultured *in vitro* have been transfected by lipofection with the plasmid pcDNA3 containing an antisense 3'-cDNA segment of rat glutaminase that is well conserved in all known glutaminase sequences. If our working hypothesis is correct, that is, that glutaminase is essential for cell-transformed phenotype sustenance, the inhibition of glutaminase by producing a glutaminase antisense RNA must result in a decrease in the proliferation rate and other characteristics of the transformed phenotype. The presented results confirm this hypothesis and the relevance of glutaminase in maintaining the malignant phenotype of tumour cells. Furthermore, transfected cells lost their tumourigenic capacity *in vivo* when inoculated in isologous mice.

MATERIALS AND METHODS

Construction of plasmids expressing antisense-RNA glutaminase

Two partial-length rat kidney glutaminase cDNA segments, HindIII (1893)–EcoRI (1614) and EcoRI (3314)–EcoRI (1614), obtained from the pGA104 recombinant plasmid [15], were subcloned in their antisense orientations behind the cytomegalovirus promoter of the pcDNA3 vector from Invitrogen (San Diego, CA, U.S.A.) by standard recombinant techniques. The resulting constructions were designated pcDNA3-0.28 and pcDNA3-1.7, respectively. The structures of the recombinants were verified by restriction-enzyme digestion of isolated DNA. The 3'-end sequences of 1.7 and 0.28 kb were selected because of the high sequence similarity found amongst cDNAs coding for C-terminal ends of known glutaminases [9,15–17]. The antibodies

Abbreviations used: EATC, Ehrlich ascites tumour cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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against renal glutaminase and the pGA104 plasmid were kindly supplied by Professor N. P. Curthoys (Colorado State University, Fort Collins, CO, U.S.A.).

Cell culture and transfection procedure

The Ehrlich ascites tumour cell (EATC) line from the American Type Culture Collection, CCL-77, and its derivatives after transfection were grown in adherent cultures in RPMI-1640 medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10 % fetal calf serum and antibiotics. The cultures were incubated in a humidified atmosphere at 37 °C with 5 % CO₂/95 % air. The culture medium for cell lines containing the neomycin-resistance gene was supplemented with 600 µg/ml G418. The plasmids or the vector control pcDNA3 were transfected in EATCs by lipofection using the lipid Dosper (Boehringer Mannheim, Mannheim, Germany). Cells were selected after growing in complete RPMI-1640 medium containing 600 µg/ml G418 for 2–3 weeks. Following G418 selection, 12 drug-resistant (neo⁺) clones (six from each construction transfected) were picked randomly from different plates and studied after expansion. As a control, six neo⁺ clones were picked from EATCs transfected with the pcDNA3 vector lacking the antisense sequences (vector-control cells).

Growth rates

Cells (10⁴ cells/3 ml of medium) were seeded in each well of a six-well plate and allowed to grow for different times. The trypsin-treated cells were counted on a haemocytometer or using a Coulter counter (Coulter, Luton, Beds., U.K.). Cell lines expressing the antisense glutaminase RNA segments, cells transfected with the empty vector, and the parental EATC line were studied. Growth assays were determined by cell enumeration in triplicate for each cell line on every day of culture up to the seventh day. Doubling times and saturation densities were calculated by standard methods. All experiments were repeated at least three times and gave similar results. Cell growth rates were also determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method described by Mossman with slight modification [18].

Colony-forming assays

Cells were seeded in triplicate with 250 cells/10-cm dish in complete medium. After 15 days of growth, the cells were fixed and stained with 5 % Giemsa stain, and the grossly visible colonies were counted. All experiments were repeated at least three times and gave similar results.

Blotting assays

Glutaminase protein in total cell extracts of transfected and non-transfected EATCs were subjected to Western-blot analyses. Exponentially growing cells (60–80 % confluence) were pelleted by centrifugation, washed twice with ice-cold PBS, and lysed on ice in RIPA buffer, pH 7.6 [30 mM Hepes, 150 mM NaCl, 1.0 % (v/v) Triton X-100, 0.1 % (w/v) SDS and 1.0 % (w/v) deoxycholic acid], with added proteinase inhibitors, pepstatin A (1.0 µg/ml), leupeptin (10 µg/ml) and 0.2 mM PMSF. Lysates were clarified by centrifugation at 10⁴ g for 10 min at 4 °C. Cell protein (25 µg/lane) was electrophoresed on 10 % SDS/polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were first incubated with a rabbit anti-rat renal glutaminase polyclonal antibody and then with a goat anti-rabbit IgG–biotin and avidin–peroxidase antibody; the

anti-rat glutaminase polyclonal antibodies recognize Ehrlich ascites cell glutaminase [10]. Glutaminase bands were detected using diaminobenzidine as a substrate (Sigma).

Northern-blot analyses were performed as described elsewhere [8] using the 0.28 kb rat kidney cDNA segment, mentioned above, as a probe.

Glutaminase assay

Phosphate-activated glutaminase activities were determined as follows: harvested cells were washed three times with 0.1 M Tris/HCl, pH 8.0, and suspended in the same buffer for 3 h in order to deplete endogenous substrates. The reaction medium was 0.1 M phosphate, 20 mM glutamine, 20 µg/ml oligomycin and 1.2 µg/ml antimycin A, to prevent mitochondrial glutamate oxidation. Under these conditions glutamine and phosphate are at saturating concentrations [19]. For each assay 0.2 ml of cell suspension was used, equivalent to 10⁶ cells; after 6 min of incubation at 37 °C the reaction was stopped by the addition of 0.5 ml of 0.4 M HCl. The KOH-neutralized mixture was centrifuged at 16000 g for 5 min. Finally, the glutamate formed was determined by the method according to Lund [20]. To be sure that glutaminase was indeed the assayed enzyme, the results obtained using controls without phosphate were always subtracted. Blanks with samples omitted were also run in parallel.

Tumourigenicity assay

To assess tumourigenic capacity, the transfected cDNA glutaminase antisense 0.28AS-2 line was intraperitoneally inoculated in isologous Swiss albino female mice of approx. 25 g in weight. Tumour growth was followed by the daily weighing of each animal [1]. The mice were housed in a controlled-environment animal facility and allowed free access to food and water.

RESULTS

Growth characteristics and morphology of the glutaminase-antisense-RNA-expressing cells

Transfection with an antisense glutaminase cDNA allows the blockage of gene expression [21]. Ehrlich ascites carcinoma cells growing *in vitro* have been transfected by lipofection, with two different genetic constructions (pcDNA3-1.7 and pcDNA3-0.28), as described in the Materials and methods section. After transfection, 12 different cell clones were selected (six from each construction transfected), and their cell-proliferation rates were determined for each clone by cell enumeration in a Coulter counter. EATC line and cell clones transfected with the pcDNA3 vector without any insert were used as controls. All transfected clones with the antisense glutaminase cDNA segments (1.7 and 0.28 kb) showed a decrease in growth rate. This decrease was more pronounced in cell lines transfected with the shorter 0.28 kb segment of glutaminase antisense cDNA (0.28AS cells). Figure 1 shows the average growth curve of 0.28AS cells, which had a growth rate approx. 50 % less than that of the controls. Colorimetric analyses of tetrazolium-stained cells provide a reliable approximation of cell-proliferation capacity [22,23]; the data obtained using the MTT test confirmed the above-expressed results by cell enumeration.

For the following growth-related experiments one of the 0.28AS cell lines, named 0.28AS-2, was selected. The main growth parameters of the 0.28AS-2 line are displayed in Table 1. The decrease in both proliferation and plating efficiency is noteworthy. This clone displayed a remarkable change in mor-

Figure 1 Growth curves of the 0.28AS clones expressing an antisense rat kidney RNA glutaminase segment compared with controls

EATC indicates the parental cell line; vector-control indicate cells transfected with the pcDNA3 vector without insert. Each point in the parental line is the mean of three different experiments; S.D. bar sizes are smaller than the symbols. Each point for vector-control and 0.28AS cells is the mean $\pm$ S.D. of six different lines; all experiments were carried out in triplicate.

Table 1 Growth characteristics of cDNA antisense glutaminase 0.28AS-2 transfected cells

EATC, parental cell line; vector-control, EATC line transfected with pcDNA3 vector without insert; 0.28AS-2, EATC line transfected with pcDNA3-0.28 construction. Saturation density represents the number of cells at saturation density per 35-mm diameter plate. Values shown are means $\pm$ S.D. Experimental differences between 0.28AS-2 cells and the two controls were analysed by the Mann-Witney U test; * $P < 0.05$.

Characteristics	EATC	Vector-control	0.28AS-2
Doubling time (h)	28.5 $\pm$ 1.5	28.9 $\pm$ 2.0	35.4 $\pm$ 0.8*
Saturation density ($\times 10^6$)	2.7 $\pm$ 0.1	2.7 $\pm$ 0.1	2.3 $\pm$ 0.1*
Plating efficiency (%)	44 $\pm$ 1.0	38 $\pm$ 2.0	26 $\pm$ 2.0*

Figure 3 Tumour growth *in vivo*

Tumour growth was determined by normalized weights of inoculated animals. Normalized weights are displayed as W_d/W_0 (W_d , weight at day d ; W_0 , weight at day 0). $\blacktriangle$, Mice inoculated with 10^7 EATC parental cells. $\blacksquare$, Mice inoculated with 10^7 vector-control-transfected cells. $\circ$, Mice inoculated with 10^7 0.28AS-2 antisense-glutaminase-transfected cells. $\bullet$, Mice inoculated with 2.5×10^7 0.28AS-2 antisense-glutaminase-transfected cells. Each point is the mean $\pm$ S.D. for six animals. Vertical arrows indicate the day on which all the animals in that particular cohort died; horizontal arrows indicate that animals survived. Similar results were obtained with animals inoculated with up to 4-fold antisense-glutaminase-transfected cells.

phology, compared with control cell lines (Figure 2): cells became polygonal and formed adherent clumps, in contrast with the random growth pattern of the controls. The 0.28AS-2 line also showed a significant decrease, about 40%, in its cloning efficiency, and the duplication time was greater than the controls, the EATC parental line and the cells transfected with the pcDNA3 empty vector.

Figure 3 displays the results of experiments on tumourigenicity *in vivo* using the 0.28AS-2 cells, compared with the control lines.

Figure 2 Optical microscopy

EATC parental cell line (A); vector-control (B) and 0.28AS-2 antisense glutaminase-transfected cell line (C). Identical magnifications were used for each line.

Figure 4 Glutaminase activity versus growth time of three different 0.28AS clones expressing antisense rat kidney glutaminase segment compared with controls

Each point is the mean $\pm$ S.D. for three different experiments. mU, m-units.

Figure 5 Western-blot analyses showing the decrease in glutaminase protein after expression of antisense 0.28 kb cDNA glutaminase segment

Lane 1, EATC parental cell line; lane 2, vector-control cell line; lane 3, 0.28AS-1 antisense-transfected cell line; lane 4, 0.28AS-2 antisense-transfected cell line; and lane 5, 0.28AS-3 antisense-transfected cell line. PAG, phosphate-activated glutaminase.

Mice inoculated with 10^7 control-line cells developed ascitic tumours and died after 16 ± 1 days; on the contrary, mice inoculated with the same number of antisense-expressing cells did not develop any tumours. Tumour growth *in vivo* was estimated by following the increase in total animal weight daily [1]. When animals were inoculated with a higher number of antisense-glutaminase-transfected cells, they only showed an increase in total weight in the first few days after inoculation due to the presence of ascitic fluid, but no cellular growth was observed. Several days later the ascitic fluid disappeared and the animals recovered their normal weight. None of the animals inoculated with antisense-glutaminase-transfected 0.28AS-2 cells showed any tumours, and they survived for several months.

Phosphate-activated glutaminase activity in antisense-transfected cells

Phosphate-activated glutaminase activities were determined in transfected and non-transfected cells during cell growth. The glutaminase activity profiles versus time obtained for cultured cells *in vitro* were similar to those obtained *in vivo* in intact ascites carcinoma cells growing in mice [8]. Glutaminase activity reached its maximum during the exponential phase of growth, with a subsequent decrease at the stationary phase of growth. Figure 4

shows the glutaminase activities versus time of three 0.28AS lines compared with the controls. The activities were similar in both controls used, but all transfected cells with the 0.28 kb antisense-cDNA glutaminase insert showed significant decreases in glutaminase activities. Western-blot analyses of these three 0.28AS lines, using polyclonal anti-rat renal glutaminase antibodies, showed a clear shortage of the glutaminase protein (Figure 5), consistent with the shortage in glutaminase activity. Non-significant changes were observed in glutaminase mRNA levels in Northern-blot experiments.

DISCUSSION

The scope of the present work was to confirm the critical role played by phosphate-activated glutaminase in the growth and proliferation of tumour cells [12,13]. The results presented here demonstrate that the stable expression of heterologous, but highly conserved [16,17], rat glutaminase cDNA antisense segments in mouse EATCs leads to a decrease in the proliferation capacity *in vitro*. After screening the behaviour of different stably transfected cell clones, the one named 0.28AS-2 was selected for the most characteristic growth experiments. As a consequence of transfection with cDNA antisense glutaminase, the cells are impaired markedly in their growth and proliferation capacity, since they show a longer doubling time and a decrease in their saturation density and plating efficiency, parameters that are correlated with malignancy. Cells also show marked changes in their morphology.

The transfected cells also display a decrease in the levels of glutaminase protein and activity; as mentioned above, many experimental data support a direct correlation between glutaminase activity and malignancy in tumours [6,7].

These results, taken together, provide evidence that the inhibition of the expression of glutaminase in tumour cells tends to revert their transformed phenotype towards normality. Furthermore, they confirm the relevance of glutamine and phosphate-activated glutaminase, the first step in the glutaminolytic process, which is essential for maintaining the energy and nitrogen metabolism of tumour cells [24]. Recently Spittler et al. [25] have reported similar results using an opposite strategy; in fact, they have communicated that low glutamine concentrations in the culture medium induce phenotypic and functional differentiation of U937 myelocytic cells.

But, what is perhaps most remarkable is that the 0.28AS-2 cells, which are able to grow *in vitro*, lose their tumourigenic capacity when they are intraperitoneally inoculated into isologous mice. The antisense derivative cells inoculated do not develop any tumours, as the controls do. Control mice (inoculated with parental and vector-control-transfected cells) die 16 ± 1 days after inoculation; the animals inoculated with cell numbers up to 2.5-fold greater than the controls survive after several months without any apparent disease. These results indicate that phosphate-activated glutaminase is not only essential for tumour growth and progression, but that this enzyme also seems to play a critical role in maintaining the tumourigenic capacity. Furthermore, the present studies provide evidence that the inhibition of glutaminase expression in tumour cells reverts the transformed phenotype *in vitro*, and inhibits their tumourigenicity *in vivo*.

Many attempts have been made in the use of glutamine-related chemotherapy in experimental oncology and in human patients [26,27]. The usefulness of glutamine analogues [azaserine, azotomycin, DON (6-diazo-5-oxo-L-norleucine) and acivicin] is very limited [28] in anti-tumour therapy because of their toxicity and the undesirable collateral effects. The use of glutamine clearance

by exogenous glutaminase has been utilized in lymphoblastic leukaemia [29], but it is also limited. These findings open up the possibility of new approaches for the clinical use of glutamine-related therapy.

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