Glutaminase isoform expression in cell lines derived from human colorectal adenomas and carcinomas

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This paper describes some properties of glutamine oxidation and glutaminase isoform expression in cell lines derived from human colorectal adenomas and carcinomas. The slow-growing adenoma-derived cell line AA/C1, and the rapidly proliferating carcinoma cell line HT29, both required glutamine for growth. The rate of $^{14}CO_2$ production from [U-^{14}C]glutamine was faster in AA/C1 cells than in HT29 cells. Conversely HT29 cells showed faster rates of glucose oxidation and lactate production. The activity of glutaminase was 3 times higher in AA/C1 cell extracts than in extracts of HT29 cells. Glutaminase activity in the two cell lines had similar K_m values for glutamine, but the activity in AA/C1 cells had a higher $K_{0.5}$ for activation by phosphate. Glutaminase activity in extracts of both cells was inhibited by glutamate. Western blotting showed the presence, in both cell lines, of isoform(s) of glutaminase with an molecular mass of 63 kDa, intermediate between that of kidney glutaminase and liver glutaminase. PCR-based analysis showed that an mRNA species identical to the kidney-type isoform glutaminase C was present in both cell types as was an additional mRNA species identical to the liver-type glutaminase isoform from human breast tumour cells. Northern blotting using isoform-specific cDNA probes demonstrated that mRNA for both glutaminase isoforms was expressed at significant levels in both cell types. Similar results to those in AA/C1 cells and HT29 cells were obtained in two further adenoma and carcinoma cell lines respectively. These results contrast with those reported previously in hepatocyte/hepatoma model systems with respect to fuel selection, glutaminase activity and isoform expression. They also constitute the first demonstration of simultaneous expression of two glutaminase isoforms in a single cell type.

Key words: colorectal, fuel selection, glutaminase, glutamine, tumour.

INTRODUCTION

Tumour cells have an absolute requirement for glutamine as a growth substrate. Glutamine is required as a precursor for both DNA synthesis and protein synthesis, and may also be used as a respiratory substrate. In experiments where glutamine metabolism in tumour cells has been specifically compared with that in non-transformed cells of the same origin, glutamine metabolism in the tumour cells has been found to be considerably faster. This is true for human hepatocytes and hepatoma cells [1] and also for glutamine oxidation in rat kidney fibroblasts and rat fibrosarcoma cells [2].

The first reaction in glutamine metabolism is hydrolysis of glutamine to glutamate via the mitochondrial enzyme phosphate-dependent glutaminase. Two major isoforms of this enzyme have been characterized. These are known as the kidney form (K-type) which was first cloned from rat kidney [3] and is expressed in many mammalian tissues, and the liver form (L-type) [4] which was originally identified in post-natal liver. These two enzymes have different kinetic properties. Although the cDNAs encoding the two isoforms have regions of high sequence similarity, they also differ significantly elsewhere and the enzyme isoforms are the products of different genes (for a review see [5]). Glutamine metabolism is essential for tumour cell growth but there are few studies at present on glutaminase expression in tumour cells. In mouse Ehrlich ascites cells [6] and rat fibrosarcoma cells [2] an enzyme with the kinetic properties of the K-type glutaminase is expressed. Rat and human hepatocytes express the L-type glutaminase, but this is not expressed in hepatoma cell lines, which express the K-type instead [1]. Inhibition of K-type glutaminase expression by anti-sense mRNA in Ehrlich ascites cells has been shown to decrease the growth and tumourigenicity of these cells [7].

Recently cDNAs encoding two further isoforms of glutaminase have been isolated and characterized. A K-type glutaminase cDNA with a modified sequence at the 3′ end of the coding region was derived from an HT29 cDNA library by Elgadi et al. [8] and termed glutaminase C. A cDNA encoding a modified L-type glutaminase with some 70 additional amino acids at the N-terminus has been cloned from the human breast tumour cell line ZR75 by Gomez-Fabre et al. [9]. mRNA for this L-type isoform is also expressed in brain and pancreas [9,10].

Although it is clear that glutamine metabolism is important in many tumour cell types, little is known about glutamine metabolism and glutaminase isoform expression in colorectal tumour cells. The establishment of cell lines derived from colorectal adenomas and carcinomas [11] has enabled characterization of cells at pre-malignant and malignant stages of tumour progression. In the present study, aspects of glutamine metabolism and glutaminase expression in the benign slow-growing adenoma cell line AA/C1 are compared in detail with those in the rapidly growing malignant colorectal cell line HT29. The investigation is extended to other adenoma and carcinoma-derived cell lines.

MATERIALS AND METHODS

Cell Lines

Human colorectal adenoma and carcinoma cell lines were donated by Professor C. Paraskeva (Department of Pathology and Microbiology, University of Bristol, Bristol, U.K.). Details of

Abbreviations used: FBS, foetal bovine serum; K-type, kidney-type; L-type, liver-type; RT-PCR, reverse transcription PCR.

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the derivation of the cell line AA/C1 from a colorectal adenoma [11] and the cell line HT29 from a colorectal carcinoma [12] are as described previously. Details of the other cell lines used in some experiments have also been described previously [11–14].

Media and standard cell culture conditions

Cell lines were grown in 75 cm^2 polystyrene tissue culture flasks (Corning Inc., Corning, NY, U.S.A.) under standard culture conditions at 37 °C under 5 % CO_2 in air, as described by Paraskeva et al. [11]. The standard growth medium was Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum (FBS) for HT29 cells, and 20 % (v/v) FBS for AA/C1 cells, insulin (0.2 units/ml), penicillin G (100 units/ml) and streptomycin sulphate (100 μg/ml). For routine culture of cells, standard medium was supplemented with 2 mM glutamine. AA/C1 cells were grown in medium which had been conditioned in the presence of Swiss 3T3 feeder cells [11] and supplemented with 20 % (v/v) FBS. Routine passage was carried out using 0.1 % trypsin. For all experiments, cells were used during a period of 50–75 % confluency and, when necessary, cell numbers were determined using a counting chamber. For all values normalized to protein concentration, cell protein was measured using the method of Bradford [15].

Cell extraction and assays of enzyme activity

Glutaminase activity in cell extracts was measured by determination of ammonia production from glutamine using the *o*-phthaldialdehyde reagent as described previously [16]. Cell extracts were prepared following trypsinization of pre-confluent cells from flasks. The pellets of trypsinized cells were washed in 50 mM Tris/HCl, pH 7.4, centrifuged at 100 ***g*** for 2 min and then resuspended in 250 μl of the same solution and passed through a 23G needle five times to ensure cell disruption. The extracts were incubated at 37 °C in the presence of appropriate concentrations of glutamine and K_2HPO_4, at pH 8.0, in a final volume of 0.1 ml for 30 min, over which time the production of ammonia was found to be linear. All reactions were stopped by the addition of 20 μl of ice-cold 10 % (w/v) trichloracetic acid. Glutaminase activity in the presence of NH_4Cl was determined by measuring glutamate production from glutamine. Glutamate was assayed enzymically using glutamate dehydrogenase and NAD^+.

Oxidation measurements

$^{14}CO_2$ evolution from cell cultures incubated in 24-well plates with [U-^{14}C]-labelled glucose or glutamine was measured using the CO_2 capture system as described by Collins et al. [17]. Essentially $^{14}CO_2$ produced by the cell culture was trapped on KOH-soaked filters which fitted tightly on to the 24-well plate. The filters were removed and radioactivity on the filters determined by liquid-scintillation counting.

Western blotting

Cells from a T75 flask were trypsinized and pelleted. The cells were washed in PBS then centrifuged and the pellet resuspended in 100 μl of Na_2HPO_4 (50 mM), pH 8, containing protease inhibitors. A 100 μl aliquot of a 5 % solution of the detergent decanoyl *N*-methyl glucamine (Mega-10) was added and the solution clarified by centrifugation at 10000 ***g*** for 1 min. Proteins were separated by SDS/PAGE and transferred to nitrocellulose. After blocking with powdered milk, the blot was probed with a polyclonal rabbit antibody raised against rat kidney glutaminase and visualized by ECL® (Amersham International, Little Chalfont, Bucks., U.K.).

Reverse Transcription (RT)-PCR

Total RNA was extracted from colorectal tumour cell lines using Tri-Reagent (Sigma) and cDNA was synthesized using an oligo dT primer. PCR was performed using various primers. PCR products were separated by agarose gel electrophoresis, excised, then extracted using the QIAquick Gel extraction kit (Qiagen, Crawley, West Sussex, U.K.) and ligated into the pGem-T-Easy vector (Promega). Competent *Escherichia coli* XL-1 Blue cells were transfected with the ligation mixture, plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen) and the insert sequenced using M13 forward and reverse primers.

Northern blotting

mRNA was isolated from total RNA using a streptavidin magnetic particle mRNA isolation kit (Roche, Lewes, East Sussex, U.K.). mRNA was run on a formaldehyde agarose gel and blotted on to nitrocellulose. After cross-linking, the blot was probed with glutaminase isoform-specific cDNA probes. The L-type probe corresponded to nucleotides 462–1018 of the ZR75 breast tumour glutaminase sequence [9] (accession number NM 013267) and was generated by PCR using AA/C1 cell cDNA as template. The K-type probe was a 0.95 kb fragment corresponding to nucleotides 841–1791 of the human cDNA clone HK03864 [18] and was a gift from Professor J. Marquez (Department of Molecular Biology and Biochemistry, University of Malaga, Malaga, Spain). Probes were labelled with [α-^{32}P]CTP using the Rediprime labelling kit (Amersham Biosciences). Hybridization was performed at 42 °C in Ultrahyb (Ambion, Austin, TX, U.S.A.), and the membrane was washed for 2 × 5 min at 42 °C in 2 × SSPE [where SSPE contains 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA] followed by 30 min at 42 °C in 2 × SSPE /0.1 % SDS before exposure to photographic film overnight. The blot was probed first with the K-type probe, then stripped and re-probed with the L-type probe. This procedure was repeated with a probe to glyceraldehyde-3-phosphate dehydrogenase as a reference. All procedures involving kits were performed according to the manufacturers' instructions.

RESULTS

The rate of proliferation of the HT29 carcinoma cells was five times faster than that of AA/C1 cells, as measured by incorporation of [^{3}H]thymidine into DNA over a 5 day period post-seeding, and both cell lines required the presence of glutamine in the culture medium for maximum growth rates (results not shown). Glutamine is required as a precursor for DNA and protein synthesis but may also be important as an energy source. In this case, glutamine carbon is metabolized to CO_2 via the citric acid cycle, with the involvement of mitochondrial malic enzyme to convert malate into pyruvate.

The degree to which glutamine could be used as an energy source in these cell lines was assessed by determination of glutamine oxidation measured as the rate of $^{14}CO_2$ production from various concentrations of uniformly labelled [^{14}C]-glutamine. Figure 1 shows that the slow-growing adenoma cells catalysed glutamine oxidation, in the absence of glucose, at a considerably higher rate than did HT29 carcinoma cells. The converse was true for the rate of glucose oxidation which was 37.2 ± 6.2 nmol/h/mg in HT29 cells and 21.9 ± 4.2 nmol/h/mg

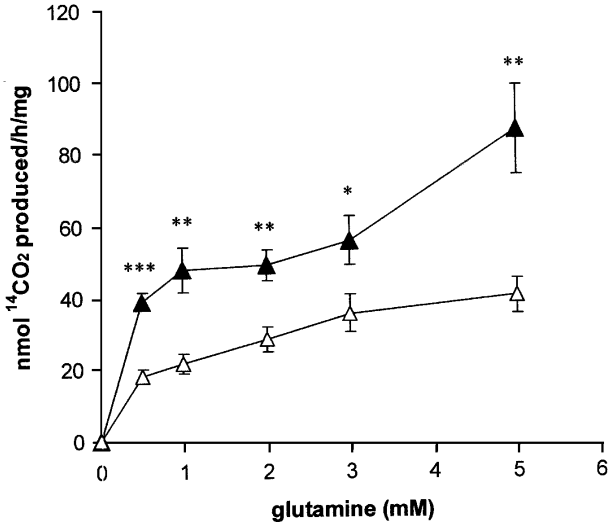

Figure 1 $^{14}CO_2$ production from [U-^{14}C]glutamine in colorectal tumour cells

Cell cultures were incubated with the concentrations of [U-^{14}C]glutamine shown for 5 h, during which $^{14}CO_2$ production was linear. $^{14}CO_2$ evolution was measured as described in the Materials and methods section. AA/C1 cells (▲), HT29 cells (△). Results shown are the mean ± S.D. of eight independent experiments. Significance was assessed by Student's *t* test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

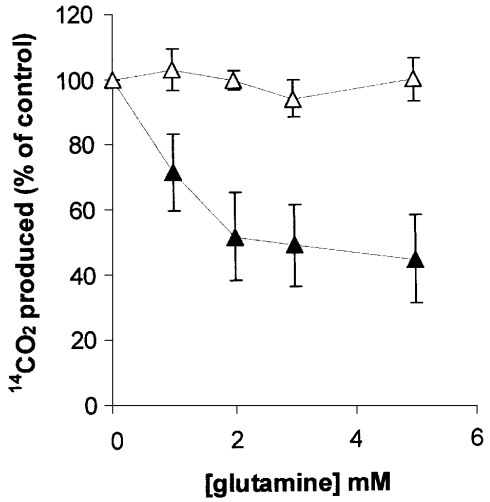

Figure 2 Effect of glutamine on $^{14}CO_2$ production from [U-^{14}C]glucose

Cells were incubated with 5 mM [U-^{14}C]glucose together with the glutamine concentrations shown for 5 h. AA/C1 cells (▲), HT29 cells (△). The control rate for AA/C1 cells in the absence of glutamine was 29.9 ± 3.2 nmol/h/mg and for HT29 cells was 67.3 ± 7.4 nmol/h/mg. Results are the means ± S.E.M. of three independent experiments performed in duplicate. All differences between corresponding values for HT29 and AA/C1 cells are significant at $P < 0.05$.

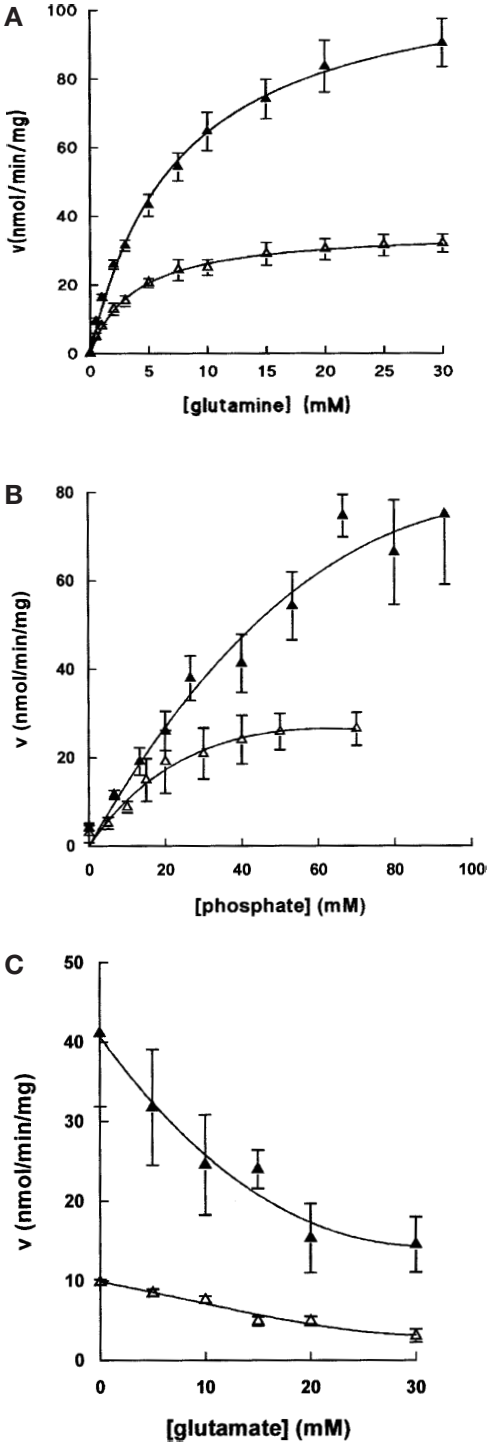

Figure 3 Kinetic properties of glutaminase in cell extracts

(**A**) Glutamine dependence. Extracts were incubated with 90 mM phosphate and the concentrations of glutamine shown. (**B**) Phosphate dependence. The glutamine concentration was 50 mM throughout. (**C**) Inhibition by glutamate. The glutamine concentration was 50 mM and the phosphate concentration was 20 mM throughout. All experiments were performed at pH 8.0 and 37 °C. Each graph shows the mean ± S.D. of single measurements from each of three separate preparations. AA/C1 cells (▲), HT29 cells (△).

in AA/C1 cells, at 5 mM glucose. HT29 cells also showed a much higher rate of lactate production from 5 mM glucose (128.5 ± 2.6 nmol/h/mg) than did AA/C1 cells (65.3 ± 6 nmol/h/mg). Figure 2 shows that $^{14}CO_2$ production from [U-^{14}C]glucose was progressively inhibited by increasing concentrations of glutamine in AA/C1 cells but not in HT29 cells. The probable interpretation of this experiment is that the release of $^{14}CO_2$ from labelled intermediates of glucose metabolism is decreased by competition with unlabelled citric acid cycle intermediates, produced from glutamine in AA/C1 cells. This effect

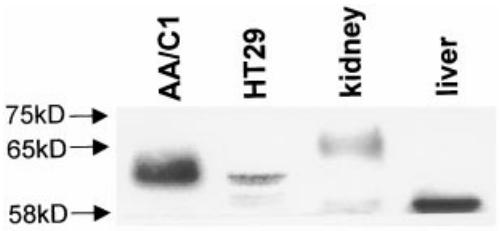

Figure 4 Western blot of glutaminase

Cell protein (50 μg) and rat liver or kidney mitochondrial extract (20 μg) were separated by SDS/PAGE, electroblotted and probed with a polyclonal antibody raised against rat kidney glutaminase. The blot is representative of six separate experiments.

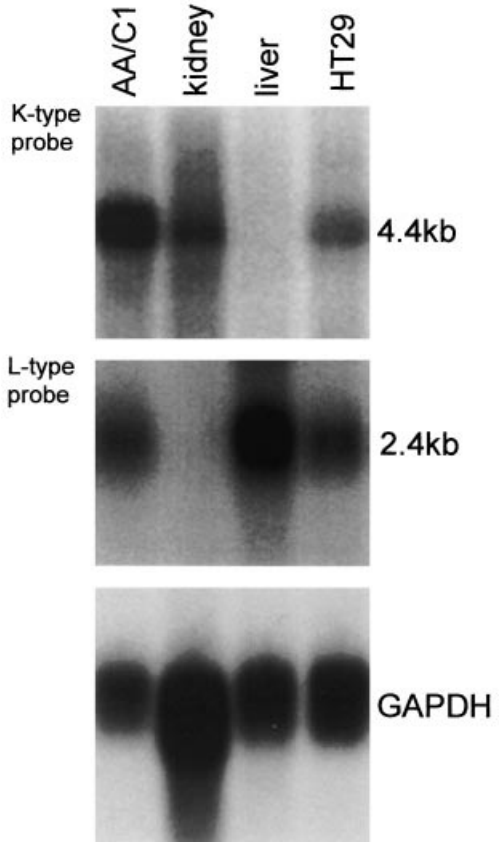

Figure 5 Northern blot of mRNA from colorectal tumour cells and rat liver and kidney

mRNA was isolated from 100 μg of total RNA in each case. The isoform specific ^{32}P-labelled probes used are described in the Materials and methods section (GAPDH, glyceraldehyde-3-phosphate dehydrogenase probe). The blot is representative of three separate experiments.

was not observed in HT29 cells where glutamine appears to be a relatively less important energy source.

Since glutamine metabolism was required for the growth of both cell lines it was of interest to characterize the properties of phosphate-dependent glutaminase, the initial enzyme in glutamine metabolism. Total glutaminase activity was measured in extracts of both cell types. Figure 3(A) shows that the K_m for glutamine was not significantly different in the two cell lines, (6.6 ± 0.5 mM for AA/C1 and 3.5 ± 0.2 mM for HT29 cells) but the V_{max} for glutaminase in AA/C1 cells was three times greater than in HT29 cells (AA/C1, 106 ± 3 nmol/min/mg; HT29, 35.1 ± 2.3 nmol/min/mg), in accordance with the observed differential rates of glutamine oxidation. The phosphate requirement for glutaminase activity in AA/C1 cells appeared to be higher than for HT29 cells (Figure 3B; $K_{0.5}$ values were 86.3 ± 27.1 mM and 25.6 ± 3.54 mM respectively). In both cell lines glutaminase activity was inhibited by glutamate (Figure 3C). The presence of ammonia did not affect glutaminase activity in either cell type, under conditions where activation of liver glutaminase is observed (results not shown).

Figure 4 shows a Western blot of extracts from both cell lines probed with a polyclonal antibody to rat kidney glutaminase which recognises all known glutaminase isoforms. The glutaminase band in AA/C1 cells was more intense than in HT29 cells, in accordance with measurements of glutaminase activity, and had the same molecular mass of approx. 63 kDa. The bands for both cell lines were clearly different in size from both rat liver glutaminase (58 kDa) and rat kidney glutaminase (65 kDa).

Because no isoform-specific glutaminase antibodies are available at present, a PCR-based approach was used to determine which glutaminase isoform was present in each cell line. Primers (forward; 5′-AAGTTTGTCATCCCTGACTTT-3′, reverse; 5′-CATACATGCCACAGGAGTGCAT-3′) to identical regions of sequence for K-type and L-type glutaminases were used in PCR with the appropriate cDNA as template. These primers flank a region which is identical in size but differs significantly in sequence between K- and L-type isoforms. Initially it was assumed that, in accordance with all the literature to date, only one isoform would be expressed in a single cell type (for a review see [5]). However, subsequent sequencing of a number of the 748 bp clones obtained showed, unexpectedly, that mRNA species for both a K-type and an L-type glutaminase were present in each cell line.

Further PCR experiments were performed to identify these isoforms. For the L-type isoform, primers were used corresponding to the ZR75 human breast cell tumour sequence [9] (accession number NM 013267). Primer pairs corresponding to the following oligonucleotides were used: (i) forward, nt 273–287 and reverse, nt 761–742; (ii) forward, nt 462–480 and reverse, nt 2098–2078; (iii) forward, nt 1984–2005 and reverse, nt 2509–2490. These primer pairs amplified products of the predicted size, which were then sequenced. The nucleotide sequence of the entire coding region was derived from these three overlapping products and was found to be 99.1 % identical with that of ZR75 cDNA.

Two human K-type glutaminases have been sequenced: human brain glutaminase and glutaminase C. These sequences are identical up to nt 1881 of the brain sequence, after which they diverge. Investigation of the K-type glutaminase from colorectal tumour cells was limited by difficulties in amplification of the highly GC-rich 5′ end of the coding region. The following primer pairs were used based on the human brain glutaminase sequence [19] (accession number AF32 7434): (i) forward, nt 580–599 and reverse, nt 1023–1001; (ii) forward, nt 883–902 and reverse, nt 1630–1611. Both primer pairs gave products of the expected size. Another forward primer was used corresponding to nt 1491–1512 of the human brain sequence, which is identical to nt 1505–1526 of the glutaminase C sequence [8] (accession number AF 158555). This primer failed to give a product when used in PCR with a number of reverse primers corresponding to the 3′ end of the coding region of the human brain glutaminase sequence. However, a 531 bp product was obtained when this primer was used with a reverse primer corresponding to nt 2038–2018 of the glutaminase C sequence. These three overlapping PCR products were sequenced. The derived cDNA sequence from these products

was 99.8% identical to the cDNA for glutaminase C over the region sequenced. This was consistent with the fact that cDNA encoding this isoform was originally cloned from an HT29 cell cDNA library.

In an attempt to determine whether mRNAs for these glutaminase species were expressed at significant levels, Northern blotting of mRNA from both cells was performed using probes specific to either L-type or K-type glutaminases. Figure 5 confirms that the probes used were specific since the K-type probe (upper panel) recognized glutaminase mRNA from rat kidney but not rat liver, and vice versa for the L-type probe (middle panel). Both cell lines expressed both mRNAs for both isoforms of glutaminase at significant levels. In both cases the K-type probe recognized a band of 4.4 kb, which is the expected size for both K-type glutaminase and glutaminase C. The L-type probe recognized a band of approx. 2.4 kb which is consistent with expression of ZR75 L-type glutaminase. Whilst it was not possible to quantify the relative levels of each isoform, the level of L-type glutaminase mRNA was consistently higher in AA/C1 cells than in HT29 cells.

The present study was extended to include two other adenoma and two other carcinoma cell lines. Maximum glutaminase activities in the adenoma cell lines BH/C1 and AA/C1/SB10C were 103.4 ± 8.1 and 123.1 ± 19.8 nmol/min/mg respectively, and in the carcinoma cell lines JW/2 and SW620 were 50.7 ± 7.2 and 68.6 ± 6.6 nmol/mg/min respectively. In all these cell lines a single band of approximately 63 kDa was obtained following SDS/PAGE and Western blotting probed with a glutaminase antibody. Northern blotting demonstrated that all the above cell lines expressed both K-type and L-type glutaminase mRNA isoforms (results not shown).

DISCUSSION

This paper describes, for the first time, some characteristics of glutaminase metabolism and glutaminase isoform expression in colorectal tumour cell lines. These results are quite different from those in previous investigations of hepatocytes and hepatoma cells. In non-proliferating hepatocytes, relatively low levels of the liver L-type glutaminase are expressed and glutamine is used mainly as a substrate for urea synthesis. Hepatoma cell lines show rapid rates of glutamine oxidation and express only K-type glutaminase [1]. In contrast, glutamine oxidation was much faster in the slow-growing adenoma cell line AA/C1 than in the HT29 carcinoma cells, and the carcinoma cells made relatively greater use of glucose as an energy source. This was accompanied by a lower activity of glutaminase in the carcinoma cells. The findings in AA/C1 cells were similar to those in two other adenoma-derived cell lines, while two other carcinoma-derived cell lines behaved similarly to HT29 cells. Taken together these results suggest that increased rates of proliferation and levels of malignancy in colorectal tumour cells may be associated with a down-regulation of one or more isoforms of glutaminase and accompanied by increased glucose metabolism.

It is clear from the measurements of glutaminase activity that both AA/C1 and HT29 cells express one or more glutaminase isoforms with K-type glutaminase kinetics, in particular a K_m of approx. 5 mM and inhibition by glutamate. The kinetics of L-type glutaminase in liver differ in that this enzyme is not inhibited by glutamate, but requires ammonia as an obligatory activator and has a K_m for glutamine of 17 mM [6]. There was no evidence for the presence of an enzyme with L-type glutaminase kinetic properties in colorectal tumour cells. If such an activity were present, it would represent only a small fraction of the total activity.

The molecular mass of glutaminase, in both cell lines, as visualized by Western blotting was intermediate between that of rat kidney glutaminase and rat liver glutaminase, and this was consistent in many experiments. Compared with the predicted molecular mass values of 59.2 kDa for rat liver and 74 kDa for rat kidney glutaminase, the calculated mass values of glutaminase C and the ZR75 breast tumour L-type glutaminase derived from the published cDNA sequences are 65.5 kDa and 66.3 kDa respectively. These two isoforms would not be clearly resolved on our Western blots. Both cell lines, therefore, appeared to express either glutaminase C or the ZR75 breast cell L-type glutaminase or both proteins. It has not been shown directly that both these cDNAs encode active enzymes, and hence the kinetic properties of these isoforms are unknown. Glutaminase C mRNA is the major isoform present in TSE cells, which have exceptionally high glutaminase activity, and for this reason it has been inferred that glutaminase C encodes an active enzyme [8]. Although Gomez-Fabre et al. [9] were able to express the ZR75 breast cell L-type glutaminase protein in *E. coli*, this did not have enzyme activity, possibility due to incorrect folding. However, the L-type enzyme cloned from ZR75 cells was presumably the major enzyme expressed, since K-type specific cDNA probes did not recognize any mRNA species from these cells on Northern blots. The results in this study show that if ZR75 L-type glutaminase protein constitutes a significant proportion of total glutaminase expression in colorectal tumour cells, then this must have the kinetic properties of a K-type enzyme.

Both the adenoma cells and the carcinoma cells expressed mRNA for both glutaminase C and for the ZR75 breast cell L-type glutaminase. This is the first demonstration of the expression of both a K-type and L-type glutaminase mRNA in a single cell type, although recently both mRNA species have been found in brain and in pancreas [10] and these tissues contain a number of different cell types. The fact that the phosphate requirement was greater in AA/C1 cells than in HT29 cells may suggest that the predominant isoform expressed may be different in the two cell lines. Further investigation will require characterization of the kinetic properties of the individual isoforms, and the availability of isoform-specific antibodies. Approaches using inhibition of specific isoform expression using anti-sense mRNA or small interfering RNA will also be important.

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