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ORIGINAL ARTICLE

Three-dimensional environment renders cancer cells profoundly less susceptible to a single amino acid starvation

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Abstract Increased amino acid requirement of malignant cells is exploited in metabolic antitumor therapy, e.g., enzymotherapies based on arginine or methionine deprivation. However, studies on animal models and clinical trials revealed that solid tumors are much less susceptible to single amino acid starvation than could be expected from the in vitro data. We conducted a comparative analysis of the response of several tumor cell lines to single amino acid starvation in 2-D monolayer *versus* 3-D spheroid culture. We revealed for the first time that in comparison with monolayer culture tumor cells, spheroids are much less susceptible to the deprivation of individual amino acids (i.e., arginine, leucine, lysine or methionine). Accordingly, even after prolonged (up to 10 days) starvation, spheroid cells could readily resume proliferation when appropriate amino acid was resupplemented. In the case of arginine deprivation, similar apoptosis induction was detected both in 2-D and 3-D culture, suggesting that this process does not determine the level of tumor cell sensitivity to this kind of treatment. It was also observed that spheroids much better mimic the in vivo ability of tumor cells to utilize citrulline as arginine precursor for growth in amino acid deficient environment. We conclude that 3-D spheroid culture better reflects in vivo tumor cell response to single amino acid starvation than 2-D monolayer culture and should be used as an integral model in the studies of this type of antitumor metabolic targeting.

Keywords Single amino acid starvation · Proliferative potential · Apoptosis · Arginine · Argininosuccinate synthetase

Introduction

Single amino acid starvation is increasingly recognized as a potentially efficient strategy for cancer treatment (Cantor et al. 2012; Wheatley 2004). A depletion of certain amino acids in the blood stream can be achieved by a specifically formulated low amino acid diet and/or by the application of specific catabolic enzymes that hydrolyze the amino acid of interest (Agrawal et al. 2012). The accelerated proliferative activity, a rapid nutritional turnover and specific alterations in the metabolic and signaling pathways make tumor cells more susceptible to amino acid withdrawal as compared with normal cells (Kuo et al. 2010). This indicates low cytotoxicity and high antineoplastic selectivity of individual amino acid starvation-based anticancer approaches and draws interest to their application in clinical practice (Scott et al. 2000).

The most successful example in this context is the application of asparaginase, the enzyme that hydrolyzes the non-essential amino acid asparagine, for the treatment of acute lymphoblastic leukemia (Müller and Boos 1998). Unlike normal cells, leukemic lymphoblasts are often

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incapable of producing their own asparagine due to a deficiency of the underlying biosynthetic pathway (Kearney et al. 2009). The lack of asparagine then causes malignant cell death (Goodsell 2005; Kearney et al. 2009).

The growing body of literature also suggests that starvation for the semi-essential amino acid arginine harbors an anticancer potential (Feun et al. 2008). Arginine auxotrophy is also caused by quantitative and/or qualitative alterations in the metabolic enzymes of the urea cycle (Dillon et al. 2004; Wheatley et al. 2005). However, we have recently shown that the in vitro susceptibility of cancer cells to arginine depletion is independent of the arginine anabolic enzymes expression (Bobak et al. 2010). Therefore, it is still unclear why some types of human malignant cells are more sensitive to recombinant arginine-degrading enzymes (e.g., arginine deiminase and arginase) than the others. The mechanisms that underlie this phenomenon are very interesting to be investigated in the prospective studies.

Up to now, the impact of single amino acid deprivation on cell survival and behavior in vitro has been studied exclusively in classical monolayer or suspension culture systems. However, we recently reported a profound loss of sensitivity of malignant cells from solid tumors to arginine deprivation when grown in a 3-D environment (Vynnytska-Myronovska et al. 2012). This finding is highly relevant to the question of the susceptibility of solid tumors to enzymotherapies targeting amino acid metabolism in vivo.

The present study was performed (1) to gain insight into the potential underlying mechanisms of the reduced sensitivity to arginine deprivation in 3-D *versus* 2-D culture, (2) to examine, if this difference in cell behavior between 2-D and 3-D environment also holds true for others amino acids in particular essential ones.

Materials and methods

Cell lines and routine culturing

Lung carcinoma A549 cells, colorectal carcinomas HT29 and HCT-116 cells (ATCC, Manassas, VA, USA) and a subline of hepatocellular carcinoma HepG2 cells (kindly provided by Prof. Kietzmann, University of Oulu, Finland) were maintained in standard Dulbecco's Modified Eagle Medium (DMEM) containing 1 g/l glucose, supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Monolayer stock cultures were kept at 37 °C in a humidified atmosphere with 8 % CO_2 in air and were dissociated for passaging and experimental setup according to a routine mild enzymatic treatment protocol using a 0.05 % trypsin/0.02 % EDTA solution for detachment. All media, media supplements and dissociation solutions were purchased from PAN Biotech GmbH, Aidenbach, Germany. Cell line origin and purity were verified by microsatellite analyses as reported in (Vynnytska-Myronovska et al. 2012). Cell lines were tested to be free of mycoplasms by means of a Mycoplasma Detection Kit MycoAlert (Cambrex Bio Science, Nottingham, Ltd, UK) and/or mycoplasma-specific PCR (Applichem, Darmstadt, Germany).

Amino acid-free media

Amino acid deprivation in culture was achieved by formulated DMEM lacking arginine (–Arg), leucine (–Leu), lysine (–Lys) or methionine (–Met). Control cells were maintained in complete DMEM (CM) which contained 84 mg/l L-arginine × HCl (0.4 mM), 104.8 mg/l L-leucine (0.8 mM), 146.2 mg/l L-lysine × HCl (0.8 mM) and 30 mg/l L-methionine (0.2 mM). All amino acid deficient and control media were supplemented with 10 % dialyzed FCS devoid of small molecules (<10 kDa), i.e., amino acids. In some experiments, the non-essential amino acid L-citrulline was added at 0.4 mM (equimolar to the concentration of arginine in complete DMEM). Survival and growth behavior in the different media compositions were monitored both in monolayer and spheroid culture.

2-D growth analyses

For monolayer growth assessment, 5×10^4 exponentially grown cells were transferred per well into 24-well plates in standard DMEM. Cells were incubated overnight to allow sufficient attachment. Then, the supernatant was removed; cells were rinsed with phosphate-buffered saline (PBS) and finally exposed to specific amino acid-free media (1 ml/well) under normal culture conditions. After defined time intervals (1–3 days), cell numbers of three wells were determined upon dissociation using a hemocytometer and Trypan Blue exclusion test. At the same time points, supernatants of three additional wells were supplemented with the appropriate amino acid to the final concentration as present in complete DMEM, and membrane-intact cells were counted 3 days later to evaluate growth restoration capacity upon amino acid starvation.

3-D formation and growth analyses

3-D spheroids were cultured using a liquid overlay technique as described previously (Friedrich et al. 2009). In brief, 500 (HepG2), 1000 (HCT-116) or 1500 (A549, HT29) cells in 200 µl of DMEM were seeded per well in 96-well plates coated with 1.5 % agarose in serum-free medium (50 µl/well). This resulted in mean spheroid sizes of 370–400 µm at day 4 in culture for all cell lines when

standard DMEM was used. 4-day spheroids were then carefully washed with PBS and transferred into new agarose-coated 96-well plates filled with either amino acid-free or amino acid-supplemented media. Single amino acid deficient conditions were maintained for a period of 10 days and supernatants were then supplemented with the respective amino acids at appropriate concentration for extended culturing in complete DMEM.

In a separate experimental series, cells were directly resuspended and seeded for spheroid formation in the different single amino acid-free media. Seeding densities and the 4-day initiation interval were identical to the standard medium approach. At day 4, the appropriate amino acids were added to the supernatant media for further spheroid monitoring.

Spheroid cultures were routinely fed twice per week by 50 % medium renewal, and spheroid integrity, morphology and size were recorded by phase contrast imaging and documented as a function of time in culture as detailed earlier (Friedrich et al. 2009; Vynnytska-Myronovska et al. 2012).

Western blot analysis

A minimum of 3×10^6 monolayer cells or 300 spheroids was collected for each control and treatment condition. Monolayer and spheroid cells were homogenized on ice with a syringe needle in lysis buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 % Nonidet P-40 and complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Protein extracts were obtained after centrifugation at 12,000 rpm for 30 min at 4 °C. Proteins were separated on a 10 % SDS-PAGE and transferred onto a PVDF membrane (Millipore Corporation, Bedford, MA, USA). Membranes were blocked in PBS containing 5 % milk powder and 0.1 % Tween-20 for 1 h at room temperature (RT) and incubated overnight at 4 °C with primary antibodies against cleaved caspase-9, cleaved caspase-8, cleaved PARP (1:1000; Cell Signaling Technology, Danvers, MA, USA) or argininosuccinate synthetase (1:2,000; BD Transduction Laboratories). After washing and 1 h incubation at RT with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (1:5000; Millipore Corporation, Temecula, CA, USA), immunoreactive proteins were visualized with an enhanced chemiluminescence system (Millipore Corporation, Billerica, MA, USA). α-Tubulin or β-actin was probed as loading controls using mouse monoclonal anti-α-tubulin (1:5,000; Millipore Corporation) or anti-β-actin (1:5,000; Sigma-Aldrich, St. Louis, MO, USA) primary antibodies.

Statistical analysis

All data are presented as mean ± SD. p values were calculated by two-sided Student's t test. The difference was considered to be statistically significant at $p < 0.05$.

Results

Apoptosis induction does not determine the level of cell sensitivity to arginine starvation

We previously applied colorectal carcinoma cells HT29 among other human epithelial cell lines as a model for the in vitro testing of arginine deprivation-based antitumor therapy (Vynnytska-Myronovska et al. 2012). The cell line was highly sensitive to arginine withdrawal in monolayer culture, but showed enhanced resistance to this kind of treatment when cultivated as multicellular spheroids. To elucidate the trigger for differential sensitivity of cancer cells to arginine starvation in 2-D *versus* 3-D culture, we compared the apoptosis induction patterns in monolayer and spheroid HT29 cells upon arginine withdrawal.

In monolayer culture, starvation for arginine induced caspase-9 activation already after 2–4 h of incubation in amino acid-free medium (Fig. 1a). The cleaved form of caspase-8 was then also detected in cells subjected to prolonged arginine depletion (72 h) (Fig. 1a). Fragmentation of poly(ADP-ribose) polymerase (PARP) is often recognized as one of the key hallmarks of caspase-dependent apoptosis (Mullen 2004). The analysis of PARP cleavage in monolayer HT29 cells upon arginine starvation revealed that its degradation strongly correlated with the time-patterns of caspases activation (Fig. 1a). These results demonstrate that arginine starvation in monolayer HT29 cells results in the activation of both mitochondrial and receptor-mediated caspase-dependent apoptotic pathways.

The analysis of PARP fragmentation in 3-D culture of HT29 cells indicated that apoptotic cell death was already taking place in HT29 spheroids of 400 μm diameter under non-starving conditions (Fig. 1b, 0-time point). This was expected from literature data showing that individual HT29 cells in the spheroid periphery of this size undergo apoptosis (Bressenot et al. 2009). However, upon spheroids cultivation in arginine-free medium the signal of cleaved PARP protein significantly increased suggesting that arginine starvation also induced apoptosis in spheroid culture (Fig. 1b). The obtained results indicate that the culture format is irrelevant for the activation of the apoptotic program and apoptosis induction may thus not be the parameter defining sensitivity and growth restoration capacity upon arginine starvation.

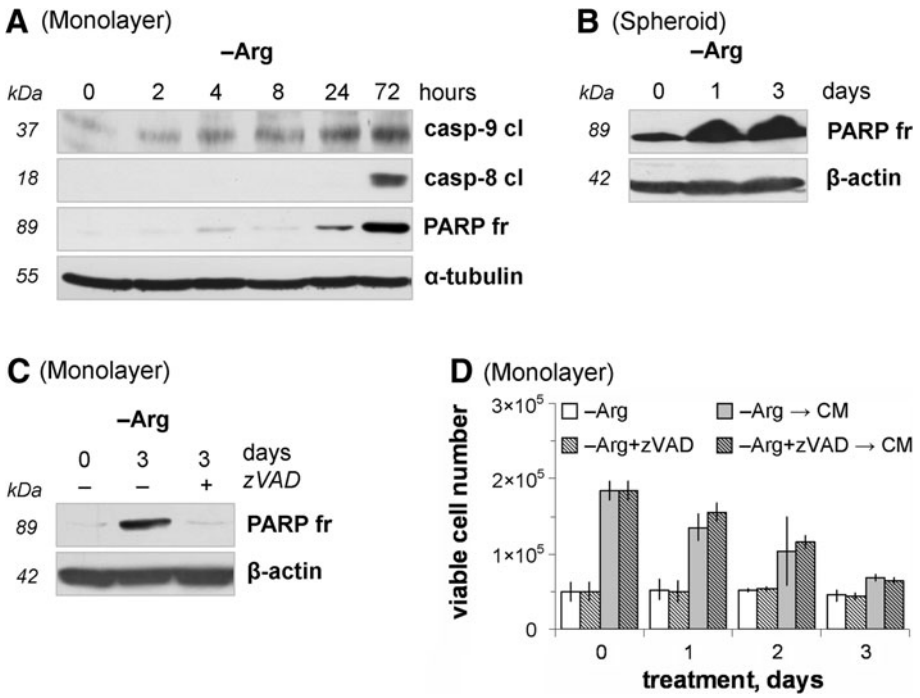

Fig. 1 Arginine starvation induces apoptosis in HT29 cells both under monolayer and spheroid culture conditions. **a** Cleaved caspase-9 (casp-9 cl), -8 (casp-8 cl) and PARP fragment (PARP fr) were determined by Western blot analysis in monolayer HT29 cells following incubation in arginine-free medium (–Arg). **b** PARP fragment was determined by Western blot analysis in spheroid HT29 cells following incubation in arginine-free medium. **c** The level of PARP fragmentation was determined by Western blot analysis in monolayer HT29 cells following incubation in arginine-free medium with or without 50 μM zVAD supplementation. **d** Monolayer HT29 cells were exposed to arginine-free medium with or without 50 μM zVAD supplementation for the times indicated and then transferred into complete culture medium (CM). Cell growth restoration capacity was analyzed as described in "Materials and methods". Each data point presents an average cell number (±SD) for three independent experiments

To prove that arginine starvation-induced apoptosis has no impact on the regrowth potential of human cancer cells, we studied the effect of *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD), a broad-spectrum irreversible caspase inhibitor, on growth restoration of monolayer HT29 cells upon arginine starvation. Our data showed that supplementation of arginine-free medium with 50 μM zVAD was sufficient to inhibit PARP fragmentation in HT29 cells (Fig. 1c). However, a time-dependent decrease of growth restoration was found for both zVAD-treated and zVAD-untreated HT29 cells starved for arginine (Fig. 1d). This result confirms our speculation that apoptosis induction does not govern the loss of growth restoration after arginine depletion, and thus does not determine the level of cancer cell sensitivity to arginine deprivation.

The ability to utilize citrulline upon arginine deprivation is enhanced in spheroid as compared to monolayer cells

We further hypothesized that arginine metabolism, in particular its *de novo* synthesis, might differ between monolayer and spheroid culture. In vivo, in addition to exogenous supply, arginine can be synthesized intracellularly from its precursor citrulline (Delage et al. 2010; Dillon et al. 2004). The key enzyme for arginine biosynthesis from citrulline is argininosuccinate synthetase (ASS). Therefore, we studied the impact of citrulline on HT29 cells growth and ASS protein expression upon arginine starvation in 2-D and 3-D culture. For this purpose, we transferred monolayer and spheroid cells to arginine-free media with or without citrulline supplementation. Citrulline was added to arginine-free medium at a concentration equimolar to arginine in complete culture medium (0.4 mM). HepG2 hepatocellular carcinoma cells were used as positive control, since they possess high ASS expression levels in monolayer culture, both under normal and arginine-deprived conditions which we had earlier shown (Bobak et al. 2010).

We observed that citrulline efficiently relieved the growth arrest of HepG2 cells in arginine-free medium in both monolayer and spheroid culture. Cell numbers (2-D culture) and spheroid volumes (3-D culture) were almost identical when grown in complete medium containing 0.4 mM arginine (CM) or in arginine-free medium supplemented with 0.4 mM citrulline (Fig. 2a). Western blot

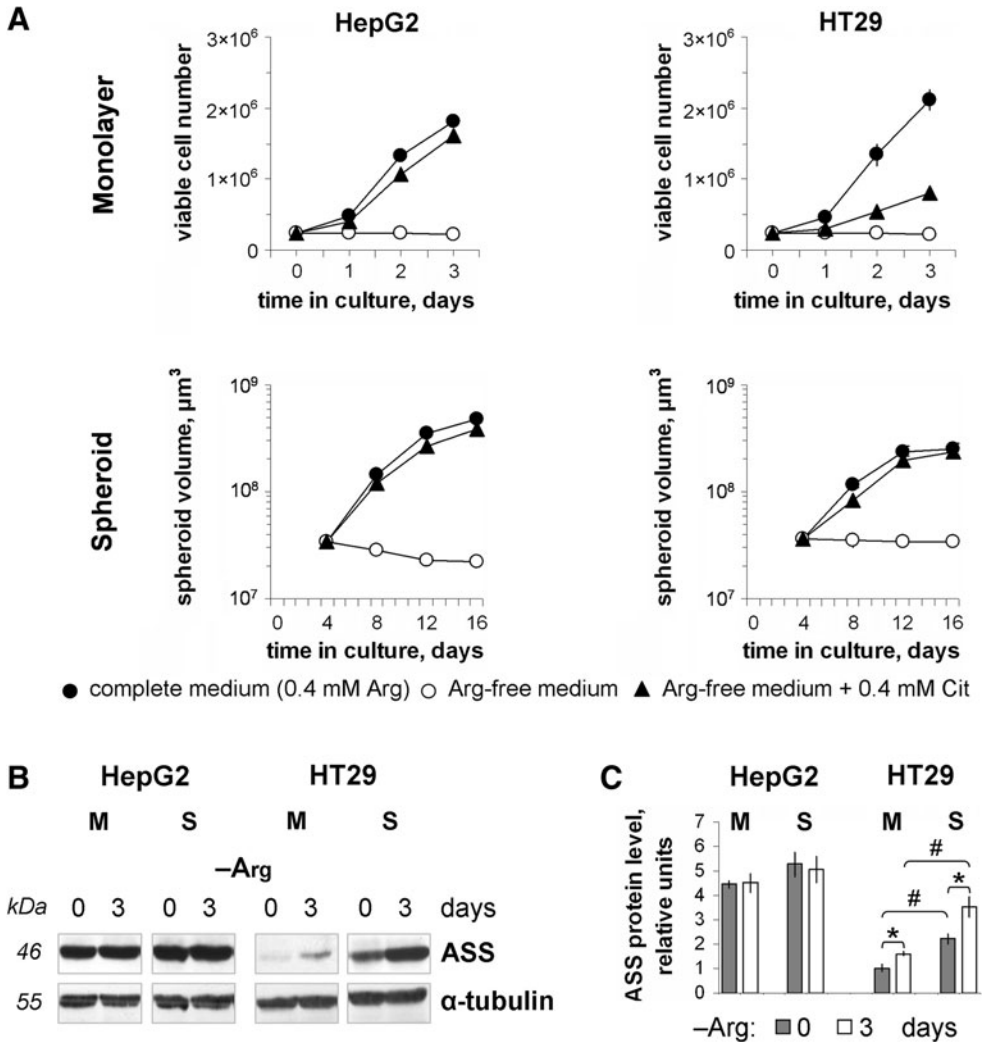

Fig. 2 Citrulline utilization and ASS expression are increased in spheroid as compared with monolayer culture. **a** Monolayer cells were seeded in complete medium at a density of 2×10^5 cells per well in 6-well plates. Cells were allowed to attach overnight, rinsed with PBS and then the medium was changed to arginine-free with or without citrulline (Cit) supplementation. At the defined time points, cell numbers were determined by Trypan Blue exclusion test. Each data point presents an average cell number (±SD) for three independent experiments. Spheroids were initiated in complete medium during 4 days. At day 4, they were rinsed with PBS and transferred to arginine-free medium with or without citrulline supplementation. Spheroid growth kinetics was assessed by monitoring spheroid volumes every fourth day during 16 days. Each data point presents the average volume (±SD) of at least 32 spheroids obtained in one experiment. Control monolayer cells and spheroids were maintained in complete medium throughout the experiment. **b** ASS protein level was determined by Western blot analysis in monolayer (M) and spheroid (S) cells following 0 or 3 days of arginine starvation (–Arg). **c** Quantification of the ASS protein level in cell samples described in Fig. 2b was done from triplicate Western blots of two independent experiments. ASS-values were compared between fed (0 days) and arginine-starved (3 days) cells (*) as well as between monolayer and spheroid cells (#). */# $p < 0.05$

analysis of lysates from HepG2 cells cultured in complete or arginine-free medium for 3 days confirmed that ASS protein expression level did not differed between the different milieu conditions or between cells maintained in monolayer or spheroid culture (Fig. 2b, c).

By contrast, monolayer and spheroid HT29 cells varied considerably in their response to citrulline supplementation in arginine-free medium. Monolayer HT29 cells only marginally grew under arginine-deprived citrulline-supplemented conditions. Conversely, the growth dynamics of arginine-starved HT29 spheroids was normalized by the presence of citrulline, i.e., spheroid volumes did no longer differ from those recorded in complete culture medium (Fig. 2a). These data are in good agreement with the observation that ASS protein expression in HT29 spheroids was much higher than in the respective monolayer cultures (Fig. 2b, c). We also established that in HT29 cells the ASS expression level was increased upon arginine deprivation when compared with non-starved cells. This effect was prominent under both monolayer and spheroid culture

conditions (Fig. 2b, c). It is of note that other cell lines characterized by low ASS level under monolayer culture conditions (A549 human lung carcinoma and HCT-116 colorectal carcinoma cells) showed essentially similar increase in ASS protein expression level in spheroid culture as HT29 cells (data not shown).

3-D environment renders cancer cells less susceptible to a lack of exogenous essential amino acids

At the level of a living organism, arginine is defined as a semi-essential or conditional amino acid (Feun et al. 2008). Under in vitro cell culture conditions, arginine has to be considered as an essential amino acid, since arginine precursors, ornithine and citrulline, are lacking in standard culture media and many tumor cells do not possess the activity of arginine biosynthetic enzyme ornithine transcarbamylase (Bobak et al. 2010). We, therefore, extended our study to evaluate whether the 3-D environment also affects the sensitivity of cancer cells to the individual deprivation for other essential amino acids (leucine, lysine or methionine). In this experimental setup, we utilized HepG2 and A549 cells as the models.

We first monitored the impact of amino acid deprivation on spheroid formation. Single cell suspensions of A549 and HepG2 cells were prepared in four different media devoid of arginine, leucine, lysine or methionine, respectively, and identical cell numbers were seeded per well into agarose-coated 96-well plates. Control spheroids were initiated in complete medium and cultures were monitored at defined time intervals. Starvation for each of the four amino acids of interest interfered with normal-size spheroid formation, but in most cases did not preclude from cell aggregation into small clusters. However, when the deficient media were supplemented with appropriate amino acids to provide complete culture conditions, cell clusters started to form compact spheroids which, despite some growth delay, increased in size according to control spheroids (Fig. 3). Essentially, the same observations were made for HT29 human colon cancer cells (Online Resource 1).

Next, we compared the influence of starvation for arginine, leucine, lysine or methionine on growth, viability and proliferative potential of monolayer cells *versus* spheroids preformed under regular culture conditions. As expected, in monolayer culture withdrawal of each individual amino acid arrested the growth of both A549 and HepG2 cells. However, survival varied between cell samples starved for different individual amino acids. In media deficient for arginine, leucine or lysine, the number of membrane-intact cells remained largely unaltered over a period of 3 days. In contrast, the number of viable A549 and HepG2 cells in the medium devoid of methionine decreased in a time-dependent manner indicating that the

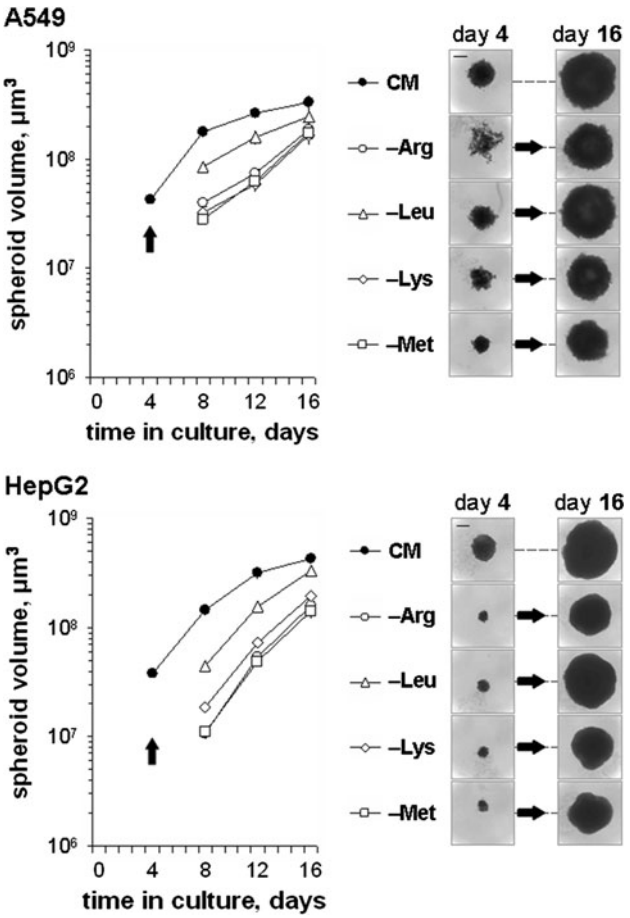

Fig. 3 Amino acid starvation interferes with normal-size spheroid initiation but does not prevent spheroid volume growth upon amino acid supplementation. Spheroids were initiated in the appropriate amino acid-free media during 4 days. At day 4, the deficient media were supplemented with the missing amino acids and spheroid growth dynamics was assessed by monitoring spheroid volumes every fourth day during the following 12 days. The time of amino acids supplementation is marked with the *black arrow*. Each data point presents the average volume (±SD) of at least 32 spheroids obtained in one experiment. Representative phase contrast images are shown to document spheroid volume directly after initiation in the deficient medium (day 4) and 12 days after the appropriate amino acid supplementation (day 16). Magnification, ×10 objective. *Bar* = 200 µm. Control spheroids were constantly maintained in complete medium (CM)

cells of interest are most susceptible to a lack of this essential amino acid (Fig. 4, white columns).

To compare the regrowth capacity after transient amino acid starvation, we analyzed the ability of monolayer cells to restore growth after different periods of amino acid withdrawal. We found that the absence of arginine, leucine or lysine for up to 3 days did not substantially affect the growth restoration capacity of monolayer A549 cells. By contrast, methionine withdrawal caused a significant decrease in cell regrowth potential already after 2 days of starvation (Fig. 4, gray columns). At the same time,

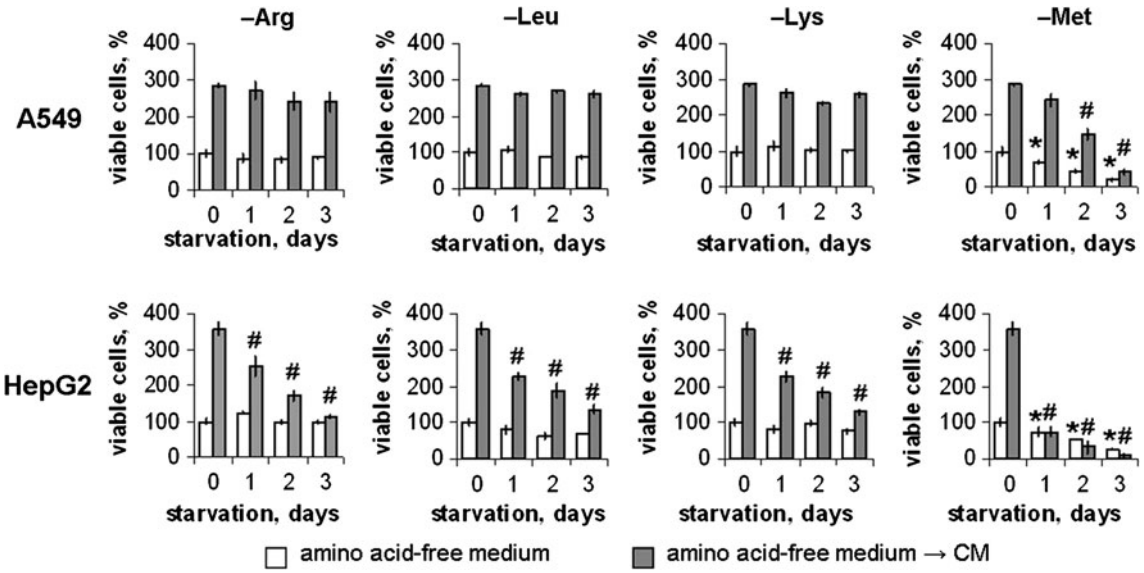

Fig. 4 The level of sensitivity to individual amino acid starvation in monolayer culture varies between different human cancer cell lines. Monolayer cells were incubated in the appropriated amino acid-free media and their growth restoration capacity in complete medium (CM) was analyzed as described in "Materials and methods". Viable cells percentage was calculated as a ratio of a viable cell number determined at a certain time point to a number of cells seeded per well, multiplied by 100. Each data point presents an average value (±SD) for three independent experiments. Cell numbers directly after amino acid deprivation and 3 days after the appropriate amino acid supplementation were compared with corresponding data of 0-time starvation. */#$p < 0.05$

monolayer HepG2 cells exhibited decreased growth restoration already after 1 day of starvation for arginine, leucine or lysine. In the case of methionine depletion, no growth recovery was observed (Fig. 4, gray columns). Therefore, in monolayer culture, HepG2 cells are in general much more sensitive to amino acid depletion than A549 cells, but methionine appears to be the most delicate essential amino acid for both cancer cell types.

We then monitored A549 and HepG2 cells susceptibility to single amino acid starvation in spheroid culture. In this setup, A549 and HepG2 spheroids were initiated in complete medium and then transferred to culture media devoid of one of the four amino acids of interest at day 4 in culture, i.e., when they had reached an average size of 370–400 μm in diameter. Starvation for arginine, leucine, lysine or methionine had no impact on spheroid integrity but abrogated spheroid growth (Fig. 5). The analysis of spheroid regrowth potential upon appropriate amino acid supplementation, however, revealed that although a growth delay was observed for starved cells in comparison with control ones, both A549 and HepG2 spheroids recovered growth even after 10 days of starvation (Fig. 5). According to the observation in monolayer culture, spheroid cells initially appeared to be most sensitive to the lack of methionine as compared to the other amino acids. This could be judged from the most prominent volume growth delay observed for spheroids incubated in methionine-free medium. Interestingly though, spheroid growth restoration occurred in all of the treated spheroids showing that spheroid cells indeed survive under these metabolic stress conditions. Taken together, our data demonstrate that human cancer cells preserve both the proliferative potential and the ability to form multicellular spheroids even after prolonged starvation for individual essential amino acids.

Monolayer cell cultures require only a single short-term (1 min) PBS-rinse to eliminate amino acids for subsequent exposure to amino acid-free medium. Spheroid cultures cannot be handled accordingly because supernatants cannot be entirely removed with one step of pipetting without inducing artifacts. To prove that the enhanced resistance of cancer cells to amino acid starvation in spheroid cultures is not due to an insufficient rinsing procedure, we monitored the ability of HepG2 spheroids to restore growth after 10 days of arginine deprivation when short-term (10 min) or long-term (1 h) PBS-rinsing procedures were applied prior to exposure to amino acid-free media. We could reveal that extended washing did not impair the regrowth potential of the tested spheroids (Online Resource 2).

Discussion

Spheroid culture systems are a sophisticated toolkit for in vitro antitumor therapy testing and are highly recommended for the identification and selection of the most promising novel treatment strategies before turning into whole animal models (Kunz-Schughart et al. 2004). Multicellular tumor spheroids mimic some essential

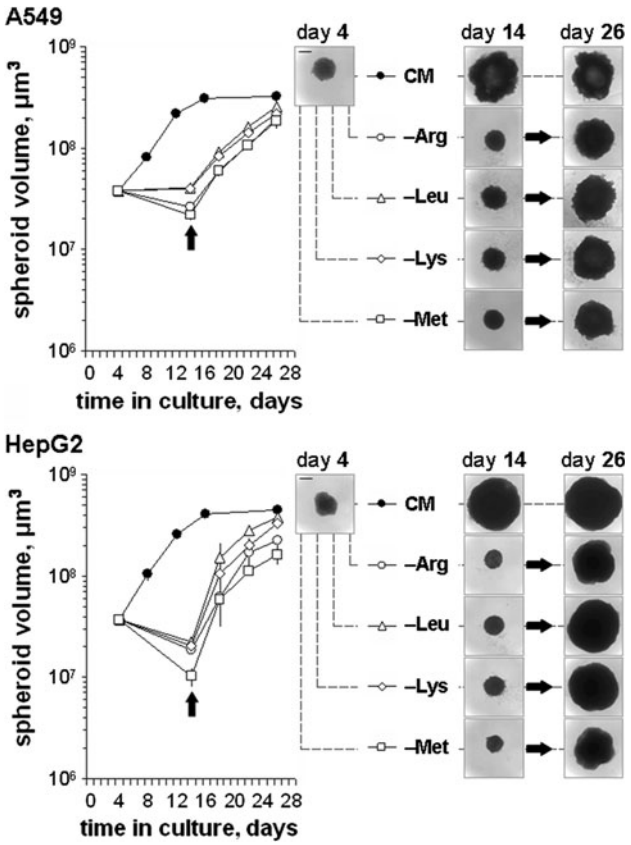

Fig. 5 Prolonged amino acid starvation inhibits spheroid growth but does not cause spheroid disintegration or prohibit growth restoration after amino acid supplementation. Spheroids were initiated in complete medium during 4 days. At day 4 they were transferred to the appropriate amino acid-free media. Spheroids were starved for each amino acid during the following 10 days. After 10 days of starvation, the deficient media were supplemented with the appropriate amino acids and spheroid growth dynamics was assessed by monitoring spheroid volumes every fourth day during the following 12 days. The time of amino acids supplementation is marked with the *black arrow*. Each data point presents the average volume (±SD) of at least 32 spheroids obtained in one experiment. Representative phase contrast images are shown to document spheroid volume directly after initiation in complete medium (day 4), after 10 days of amino acid starvation (day 14) and 12 days after the appropriate amino acid supplementation (day 26). Magnification, ×10 objective. *Bar* = 200 µm. Control spheroids were constantly maintained in complete medium (CM)

pathophysiological characteristics present in microregions of solid tumors and avascular micrometastases. Unlike in monolayer, spheroid cells develop 3-D cell-to-cell contacts, extracellular matrix assembly and proliferative patterns that more closely reflect the in vivo situation (Friedrich et al. 2007; Hirschhaeuser et al. 2010). Accordingly, proteome analyses of spheroid *versus* monolayer cells revealed differences in the expression profiles of several protein groups linked to cell proliferation control, stress response regulation and multidrug resistance phenotype (Gaedtke et al. 2007; Kumar et al. 2008; Poland et al. 2002). Moreover, cell response to the treatment in multicellular spheroids in contrast to 2-D assays is affected by a more in vivo-like 3-D penetration, binding, uptake and activity pattern of the putative drug candidates (Hirschhaeuser et al. 2010). Because of the morphological and functional properties, spheroids are considered to better reflect the tumor cell behavior than traditional monolayer cultures and to be a robust test system for in vitro remedy screening. There are casual studies exploiting multicellular tumor spheroids as a tool to investigate tumor cells radiation responses and chemosensitivity to potential anticancer agents (Ivascu and Kubbies 2006; Schwachofer 1990). Prior to our studies, no one applied spheroids as a testing platform for the investigation of the antitumor potential of metabolic approaches, in particular those targeting single amino acid metabolism.

Metabolic targeting of amino acids is a promising, yet challenging strategy for anticancer therapy. Systematic studies to unravel the mechanism of the response of malignant cells from solid tumors to this potential treatment option have mainly used monolayer culture assays as readout. Arginine deprivation is considered to be one of the most promising approaches in metabolic anticancer treatment. In vitro studies suggest that a broad range of the malignant cell types are potential targets of arginine starvation-based strategy. This includes metastatic melanomas and hepatocarcinomas, renal cell carcinomas and prostate cancer cells and mesotheliomas (Ascierto et al. 2005; Izzo et al. 2004; Kim et al. 2009; Yoon et al. 2007; Szlosarek et al. 2006). Several reports showed that arginine withdrawal in monolayer and suspension culture causes a substantial loss of viability of various cancer cell types (Cheng et al. 2007; Gong et al. 2000; Hsueh et al. 2012; Scott et al. 2000; Vynnytska et al. 2011). However, the first phase I/II clinical trials of arginine-depleting enzyme arginine deiminase showed a tumor response rate ranging from 0 to 47.4 % (Ascierto et al. 2005; Glazer et al. 2010; Izzo et al. 2004; Yang et al. 2010). To shed some light on the apparent discrepancy between the results of the in vitro experiments and clinical studies, we recently implemented a spheroid culture model for the investigation of the antitumor potential of this single amino acid depletion-based approach and observed an essential loss of sensitivity of cancer cells to the deprivation for arginine when grown in 3-D spheroid environment (Vynnytska-Myronovska et al. 2012). The exceptional therapeutic relevance of this finding motivated us to gain further insight into this phenomenon and provided the rational for studying the impact of exogenous essential amino acids (e.g., leucine, lysine, or methionine) on cancer cell survival and growth in 2-D monolayer *versus* 3-D spheroid culture. We demonstrated that although in monolayer culture tumor cells were rather susceptible not only for arginine starvation, but also for the

deprivation of other amino acids, e.g., methionine, tumor spheroids preserved regrowth potential within prolonged depletion of all tested amino acids. These findings suggest that an increased resistance of spheroids *versus* monolayers to single amino acid starvation is not an exception revealed for arginine, but rather a stable characteristic of this type of cell culture that is repeated for different essential amino acids.

There are some evidences that arginine deprivation in addition to cell growth arrest induces programmed cell death in monolayer culture (Kim et al. 2009; Lam et al. 2011; Savaraj et al. 2010). We also observed enhanced apoptosis in monolayer tumor cells exposed to arginine-free culture conditions (Vynnytska et al. 2011). However, we were surprised to detect similar apoptosis induction in spheroid culture upon arginine starvation despite the increased resistance to arginine deprivation with respect to overall survival and regrowth capacity. These results, together with the finding that in monolayer culture pan-caspase inhibitor zVAD upon arginine depletion inhibited apoptotic manifestations but failed to improve the regrowth potential of tested cells, suggest that intrinsic characteristics of the surviving cells rather than the proportion of cells undergoing apoptosis determine the sensitivity of solid tumors to arginine starvation.

Another aspect that may contribute to the discrepant sensitivities of cancer cells to arginine starvation in vitro and in vivo relates to argininosuccinate synthetase (ASS), the key enzyme that converts citrulline to arginine. Its expression was shown to correlate with the tumor resistance to arginine-degrading enzyme arginine deiminase treatment (Feun et al. 2012; Kelly et al. 2012; Tsai et al. 2009, 2012). The screening of ASS expression has been proposed as a strategy of an individualized therapy to identify those patients who are most likely to benefit from arginine depletion-based treatment (Delage et al. 2010; Dillon et al. 2004; Feun et al. 2012). The immunohistochemical analysis of tumor biopsies revealed that ASS expression varied greatly between the tumor types and tissues of origin (Dillon et al. 2004). Melanomas, hepatocellular carcinomas and prostate carcinomas were shown to be most frequently deficient in ASS, while some human cancers were almost always positive for ASS, e.g., lung and colon carcinomas (Dillon et al. 2004). At the same time, our studies revealed that lung adenocarcinoma cells A549 cells and 9 out of 16 tested colon cancer cell lines (data not shown) in monolayer cultured are characterized by the exceptionally low level of ASS expression. To understand this disagreement between in vitro and in vivo data, we compared ASS expression between monolayer and spheroid cultures of the same cell lines. We observed an increase in the level of ASS expression under the 3-D spheroid environment in human epithelial cancer cells that are characterized by marginal level of ASS enzyme in monolayer culture. These findings suggest that when compared with 2-D culture, spheroids not only better reflect the level of tumor cell sensitivity to arginine starvation, but also the more closely mimic the peculiarities of arginine metabolism in tumor cells in vivo. Therefore, the biotool "spheroid assay" will also be useful to develop new therapeutic approaches, e.g., to down-regulate ASS activity or to combine metabolic targeting with other treatment options.

Taken together, our data suggest that in comparison with monolayers spheroids better reflect the response of human tumor cells to single amino acid depletion in vivo. Therefore, we highly recommend implementing 3-D spheroid cultures in pre-animal and pre-clinical metabolic targeting test programs.

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Conflict of interest The authors declare that they have no conflict of interest.

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