Archives of Biochemistry and Biophysics 478 (2008) 96–102

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Glutamine synthetase is essential for proliferation of fetal skin fibroblasts

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ARTICLE INFO

Article history:
Received 28 May 2008
and in revised form 2 July 2008
Available online 17 July 2008

Keywords:
Cell proliferation
Skin fibroblasts
Glutamine synthetase
Fetal period

ABSTRACT

Background. Glutamine synthetase (GS) is ubiquitously expressed in the human and plays a major role for many metabolic pathways. However, little is known about its role during the fetal period. *Methods.* Cultured skin fibroblasts derived from an aborted fetus deficient in GS activity due to a R324C exchange as well as fetal and mature controls were used to determine the level of GS-expression, apoptosis, and proliferation in presence or absence of exogenous glutamine. *Results.* Glutamine synthetase can be found at early gestational stages. Loss of GS activity either inherited or induced through L-methionine sulfoximine leads to an upregulation of the GS protein but not of the GS mRNA and results in a significant drop in the proliferation rate but has no effect on apoptosis. Exogenous glutamine does not influence the rate of apoptosis but increases proliferation rates of the fetal but not the mature fibroblasts. *Conclusion.* GS can be found during early human fetal stages when it displays a significant effect on cell proliferation.

Glutamine synthetase (GS[1]; glutamate ammonia ligase, EC 6.3.1.2) catalyzes the conversion of glutamate and ammonia to glutamine by an ATP-dependent reaction. GS is encoded by a highly conserved gene and can be considered as a key enzyme in metabolism of pro- and eukaryotes [1]. In human plasma, glutamine is the most abundant amino acid and an important source for the biosynthesis of several amino acids, purines, and pyrimidines [2]. GS plays a decisive role in ammonia and glutamate detoxification, pH homeostasis, interorgan nitrogen flux, acid–base regulation, and cell signaling [3]. GS is localized in the cytoplasm and is ubiquitously expressed in the human organism with high concentrations in liver, brain, and skeletal muscle [4]. In the liver, GS expression is confined to a small cell population at the hepatic venous outflow of the liver acinus [5–7]. In astrocytes, GS operates as a neuroprotective enzyme by removing excess of ammonia and glutamate [8]. Recently, an inherited defect of GS activity in two children of consanguineous Turkish background who both died from multi-organ failure was reported and ascribed to the exchange of R324C and R341C, respectively, in the active site [9]. Systemic glutamine deficiency was the leading biochemical finding. As the most striking clinical sign, severe brain malformation with abnormal gyration and marked white-matter lesions was observed [9].

There are only few studies on GS expression and activity as well as glutamine metabolism during fetal development. In rat liver, an atypical GS distribution pattern developing in the late fetal period was described. Before that stage, megakaryocytes showed intense staining on immunohistochemistry but liver parenchyma was only faintly stained [10]. Other than in the liver, rat astrocytes showed the appearance of GS mRNA and protein as early as on embryonic day 14 [11,12]. In a porcine model, glutamine exhibited the highest fetal:maternal plasma ratio among all amino acids, suggesting active synthesis and release of glutamine by the placenta [13]. In an ovine model, about two-thirds of glutamine transported in the umbilical vein was found to be derived from uterine uptake with one-third derived from placental production [14].

Transport of glutamine via the placental barrier is provided by System N amino acid transporter 1 (SNAT 1) that showed an increasing rate of expression in the rat placenta during the final third of gestation [15]. In line with a role of GS in fetal development, complete knockout of the mouse GS resulted in early

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[1] *Abbreviations used:* GS, glutamine synthetase; SNAT 1, System N amino acid transporter 1; GS−, fetal GS activity-deficient fibroblasts; GS+, fetal GS wild type fibroblasts; GS control, GS wild type fibroblasts from a 2-year-old child; MEM, Minimal Essential Medium; FCS, fetal calf serum; MSO, methionine sulfoximine; SDS, sodium dodecyl sulphate-polyacrylamide; GAPDH, glyceraldehyde phosphate dehydrogenase; TBST, Tris buffer saline tween; RPL, ribosomal protein L13a; cpm, counts per minute; CFDA, carboxyfluorescein diacetate; MFI, mean fluorescence intensity.

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.07.009

embryonic lethality [16]. In the same study, GS deficient embryonic stem cells exhibited a reduced survival if transplanted into wild type blastocysts and failed to survive in aggregates with tetraploid wild type blastomeres [16]. Confirming the importance of glutamine for fetal development, exogenous glutamine was shown to improve mouse embryonic development when added to the culture medium [17].

In this paper, cultured human skin fibroblasts derived from a GS activity-deficient fetus of 17 weeks of gestation were investigated under different culture conditions as a model to study the role of GS on basal growth properties in early fetal stages. Hereby, an important role of GS for the proliferation of skin fibroblasts at early fetal stages was elucidated.

Materials and methods

Skin fibroblasts

Cultured skin fibroblasts were obtained from an aborted fetus after prenatal diagnosis by molecular genetic means, which had revealed a homozygous mutation (R324C) of the GS gene. The index patient of this family suffered from congenital GS deficiency and has been reported earlier [9]. As a result of the prenatal diagnosis, the gestation was terminated at 16+4 weeks of gestational age and a fetal skin biopsy was taken. The skin biopsy was initially cultured in standard medium containing a surplus of 10 mM glutamine in order to prevent growth failure as reported before [9]. As control cells, skin fibroblasts from a fetus aged 16+6 weeks of gestation who was aborted because of a severe feto-fetal transfusion syndrome as well as skin fibroblasts from a 2-year-old child taken for other diagnostic purposes were used. For this study, cells are named GS− (fetal GS activity-deficient fibroblasts), GS+ (fetal GS wild type fibroblasts), and GS control (GS wild type fibroblasts from a 2-year-old child). All cell lines were investigated at the GS locus by DNA-sequencing of all coding exons including the flanking intronic regions as described [9].

The parents agreed to the use of the skin fibroblasts of their children for scientific purposes by written informed consent. The study was approved by the Ethics Committee of the University of Münster.

Cell culture

Cells were cultured in 10 ml supplemented Minimal Essential Medium (MEM, PAA, Pasching, Austria) containing 10% fetal calf serum (FCS, Biowest, Nuaillé, France), 1% streptomycin, 1% penicillin (Biochrome, Berlin, Germany), and 2 mM glutamine and routinely passaged twice weekly by trypsinizing and seeding at 1:4 dilution in 75 cm^2 flasks (Greiner Bio One, Frickenhausen, Germany).

For all experiments, GS−, GS+, and GS control cells were washed by PBS, trypsinized and counted using the Neubauer–Zählkammer. Cells were then plated in 96-well or 6-well plates. Culture conditions varied with respect to the concentrations of extracellular glutamine (0–10 mM) or the presence of L-methionine sulfoximine (MSO, Sigma, Taufkirchen, Germany), an irreversible inhibitor of GS [18,19].

Cerebro-cortical astrocytes from Wistar rats were cultured in DMEM as described before and used for control purposes [20].

Western blot analysis

Cells were harvested in lysis buffer containing 50 mM Tris hydrochloride (pH 7.4), 1% Triton X-100, 150 mM sodium chloride, 1 mM EDTA, and a protease inhibitor cocktail (1 tablet per 15 ml; Roche, Mannheim, Germany). The protein content was determined using the Coomassie Plus Assay Reagent (Bio-Rad, München, Germany). Proteins were analyzed by Western blotting with use of 10% SDS (sodium dodecyl sulphate-polyacrylamide) gels and a semidry transfer apparatus (Amersham Pharmacia, England). Blots were probed overnight at 4 °C with antisera against GS (1:5000 solution, Sigma–Aldrich, München, Germany) and against GAPDH (glyceraldehyde phosphate dehydrogenase, 1:10,000 solution, Biodesign International Saco, USA), respectively. All antibodies were diluted in Tris buffer saline tween (TBST, Sigma, Taufkirchen, Germany). After washing with TBST and incubation with horseradish-peroxidase coupled antirabbit IgG or antimouse IgG antibody (1:2500 solution, Dako, Cambridgeshire, England) at 20 °C for 1 h, blots were developed with the use of Western-Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, MA, USA).

Quantitative real-time PCR

To determine the level of the GS transcript in addition to the Western blot analyses cultured GS−, GS+, and GS control skin fibroblasts were taken for preparation of RNA using a standard kit (RNeasy Mini Prep, Qiagen, Hilden, Germany). This was performed twice using independent cultures of each cell line. Total RNA (1 μg) was used for reverse transcription utilizing the first strand cDNA synthesis kit and oligo-p$(dT)_{15}$ primers (Boehringer, Mannheim, Germany). The quantitative cDNAs were used to determine the level of gene expression by real-time reverse transcription-polymerase chain reaction (RT-PCR) as described previously [21]. As endogenous housekeeping genes, fragments of glyceraldehyde phosphate dehydrogenase (GAPDH) and ribosomal protein L13a (RPL) were amplified. Primers used for PCR analysis were as follows: for GS forward 5′-GCTGGTGTAGCCAATCGTAGC-3′ and reverse 5′-GGCTTCTGTCACCGAAAAGG-3′ (fragment size 122 bp); for GAPDH forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGAG-3′ (fragment size 86 bp); for RPL forward 5′-AGGTATGCTGCCCCACAAAAC-3′ and reverse 5′-TGTAGG CTTCAGACGCACGAC-3′ (fragment size 141 bp). The relative expression was calculated as $2^{\Delta C_t GS}/2^{\Delta C_t \text{Housekeeping gene}}$. All measurements were repeated two times.

Determination of cell viability

GS−, GS+, and GS control cells were cultured as described above in the presence of 0, 2, 4, 6, 8, and 10 mM glutamine for 6 days in T25 flasks at 37 °C and 5% CO_2 incubation. Then, cells were washed twice with PBS, trypsinized and centrifuged for 10 min at 600*g*. Cell pellets were solved in 400 μl standard MEM and quantified by staining with 5 μl Annexin V-FITC (PharMingen, San Diego, CA) following a 15 min incubation in darkness before analysis with FACScan flow cytometry (Becton–Dickinson, San Jose, CA). As a positive control of Annexin-V-FITC, cells were cultured for 12 h in standard medium with 1 μM staurosporine (Axxora, Lörrach, Germany).

Measurement of cell proliferation by [^{3}H]-thymidine incorporation

All cell lines (2.5×10^3/well) were plated in 96-well round-bottom microtiter plates (Nunc, Rosklide, Denmark) in triplicate. Cells were cultured for 3 days at 37 °C and 5% CO_2 under the experimental conditions described above. [3H]-thymidine incorporation was performed in a final volume of 200 μl with 1 μCi/well [^{3}H]-thymidine (Hartman Analytics, Braunschweig, Germany) that was present for the last 12 h of the experiment.

Plates were frozen at −20 °C for 2 days in order to remove the adherent cells. After thawing at 37 °C, cells were transferred to a filter pad by filter mate Harvester (Perkin-Elmer, MA, USA). Filter