De novo expression of glutamine synthetase during transformation of hepatic stellate cells into myofibroblast-like cells

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The expression of glutamine synthetase (GS) was studied in cultured quiescent hepatic stellate cells (HSC) and during their transformation into myofibroblast-like cells. GS mRNA was detectable in quiescent HSC (1-day culture); however, the enzyme protein was not expressed, as assessed by Western blot analysis, immunocytochemistry and the absence of detectable enzyme activity. Similar findings were obtained after 2 days of culture; in addition, the mRNA levels had dropped by about 70 %, but they increased again thereafter during the process of HSC transformation in culture, as indicated by the expression of α-smooth-muscle actin. In parallel with the accumulation of α-smooth-muscle actin, GS was expressed, as shown by Western blot analysis and immunocytochemistry, and enzyme activity increased from undetectable levels in quiescent cells to 0.13 ± 0.01 μmol/h per mg of cell protein within 7–14 days. This value compares with GS activity in liver parenchymal cells of 0.57 ± 0.03 μmol/h per mg of cell protein. The findings suggest that activation of HSC results in the *de novo* expression of GS protein and activity, and this may serve as another marker of HSC transformation.

INTRODUCTION

The liver plays an important role in glutamine metabolism, with implications for ammonia and bicarbonate homoeostasis (for reviews, see [1–3]). In the liver acinus, glutamine-metabolizing enzymes are heterogeneously distributed [4,5]. Whereas glutaminase is found in periportal hepatocytes, glutamine synthetase (GS) is expressed exclusively in a small subpopulation of perivenous liver parenchymal cells, and these cells are thought to scavenge the ammonia that has escaped upstream urea synthesis [3]. Immunohistochemical studies demonstrated the absence of GS from hepatic non-parenchymal cells [5]. Hepatic GS activity is strongly decreased in human liver cirrhosis [1,6] or after acute or chronic tetrachloromethane poisoning, which may be of importance for the pathogenesis of hyperammonaemic states under these conditions [7,8]. Chronic liver injury, however, also results in the transformation of hepatic stellate cells (HSC) into proliferating myofibroblast-like cells. Unlike quiescent HSC, these activated cells no longer contain vitamin A droplets, but express α-smooth-muscle actin and are a major source of extracellular matrix proteins ([9]; for reviews, see [10–12]).

Here we report on the *de novo* expression of GS in activated HSC, which may serve as another marker of HSC transformation into myofibroblast-like cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, glutamine and fetal calf serum were from Biochrom (Berlin, Germany). Nycodenz was from Nycomed (Oslo, Norway). Pronase was from Merck (Darmstadt, Germany). DNase I and collagenase were from Boehringer Mannheim. Antibodies to α-smooth muscle actin, FITC-conjugated anti-mouse IgG (whole molecule), chicken serum albumin, L-γ-glutamyl hydroxamate and propidium iodide were obtained from Sigma (Deisenhofen, Germany). Anti-(mouse GS) antibody was from Chemicon International (Harrow, U.K.). Horseradish peroxidase-conjugated anti-mouse IgG antibody was from Bio-Rad (Hercules, CA, U.S.A.). RNeasy® total RNA kit was obtained from Qiagen (Hilden, Germany). The GS cDNA fragment used for detection of GS mRNA was kindly provided by Dr. W. H. Lamers (University of Amsterdam, The Netherlands). The 1.0 kb cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used for standardization, was from Clontech (Heidelberg, Germany). Nitrocellulose membranes were purchased from Schleicher & Schuell (Dassel, Germany). [α-^{32}P]dCTP was from Amersham (Braunschweig, Germany). Polybead fluorescent microspheres (2.5 % solids latex; 1.1 μm diameter) were obtained from Polysciences Ltd. (St. Goar, Germany). All other chemicals were from Merck.

Isolation and cultivation of parenchymal cells from rat liver

Isolated parenchymal cells were prepared from livers of 5–8-week-old male Wistar rats by a collagenase perfusion technique as described [13]. Cells (1×10^6) were plated on collagen-coated culture dishes and maintained in Krebs/Henseleit medium supplemented with 6 mM glucose in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. After 2 h, the medium was removed and the culture was continued for 24 h in Dulbecco's modified Eagle's medium containing 5 % (v/v) fetal calf serum, 1 % (w/v) penicillin/streptomycin, 100 nM insulin, 100 nM dexamethasone, 30 nM sodium selenite and 1 μg/ml aprotinin.

Isolation and cultivation of HSC

HSC from 1-year-old male Sprague–Dawley rats were prepared by collagenase/Pronase perfusion and isolated by a single Nycodenz gradient, as described by others [14]. The cells were seeded at a density of 0.2×10^6 (or 0.4×10^6) cells/3.6 cm² (10 cm²) on 12 (6)-well culture plates (Falcon) and maintained in 1 ml (2 ml) of Dulbecco's modified Eagle medium containing 4 mM L-

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthetase; HSC, hepatic stellate cells.

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glutamine, 1 % (w/v) penicillin/streptomycin, 12.5 mM glucose and 10 % (v/v) heat-inactivated fetal calf serum. The culture medium was changed after 24 and 48 h. Culture was performed in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. The purity of HSC was $\geqslant$ 94 %, as assessed 24–48 h after seeding by their typical light-microscopic appearance, vitamin A-specific autofluorescence and inability to phagocytose fluorescent 1.1 μm latex particles.

Determination of α-smooth-muscle actin expression

Cells were harvested in a buffer containing 63 mM Tris/HCl (pH 6.8) and 1 % SDS. The protein content of the lysate was determined according to Bradford [15] using a commercially available reagent from Bio-Rad. The cell lysate was sonicated for 10 min and mixed 2:1 (v/v) with gel loading buffer containing 300 mmol/l dithiothreitol (pH 6.8). After boiling for 5 min, the proteins were subjected to SDS/PAGE (30 μg of protein per lane; 7.5 % gel). Following electrophoresis, gels were equilibrated with transfer buffer [39 mM glycine, 48 mM Tris base, 0.03 % SDS, 20 % (v/v) methanol]. Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. Blots were blocked in 5 % (w/v) BSA-containing 20 mM Tris, pH 7.5, 150 mM NaCl and 0.1 % Tween 20 (TBST), and then incubated at 4 °C overnight with mouse anti-(α-smooth-muscle actin) antibody (1:2000 dilution). Following washing with TBST and incubation with horseradish peroxidase-coupled anti-(mouse IgG) antibody (dilution 1:10000) at room temperature for 2 h, the blot was washed extensively and developed using enhanced chemiluminescent detection (Amersham). Blots were exposed to Kodak X-OMAT AR-5 film (Eastman Kodak Co., Rochester, NY, U.S.A.) for 1–10 min.

Determination of GS expression

GS expression was determined in the same cell lysate using the same procedure as described for α-smooth-muscle actin, but without sonication. The primary antibody used in this detection was mouse anti-GS antibody (1:1000 dilution).

Determination of GS activity

HSC were cultured on six-well culture plates (Falcon) for the time period indicated and then harvested by scraping into 50 μl of distilled water per well and homogenized by sonication for 4 s at 4 °C. The pooled cell contents of four to six wells were necessary for each determination. Parenchymal cells were cultured for 24 h and then harvested by scraping into 250 μl of distilled water per well of parenchymal cells. GS activity was measured using the method described by Ward and Bradford [16], but without Triton. Aliquots of 60 μl of homogenate were incubated with 50 μl of reaction mixture at 37 °C. The final concentrations used were as follows: 100 mM imidazole buffer (pH 7.2), 12.5 mM $MgCl_2$, 10 mM sodium ATP, 50 mM potassium glutamate, 20 mM β-mercaptoethanol, 1 mM ouabain, 13 mM phosphoenolpyruvate, 100 mM hydroxylamine (freshly neutralized to pH 7.2 with KOH) and 5 units/ml pyruvate kinase. The reaction was stopped after the time period indicated by adding 250 μl of a fresh mixture containing 500 mM $FeCl_3$, 400 mM perchloric acid and 400 mM HCl. After 20–30 min incubation on ice, the protein precipitate was sedimented by centrifugation at 20000 ***g*** and 4 °C for 10 min. The absorbance of the supernatant was measured at 492 nm. Standard curves were established using L-γ-glutamyl hydroxamate. The protein content of the homogenate was determined by the Bradford method [15] using a commercially available reagent from Bio-Rad. Chicken serum albumin was used as standard. Values are expressed as μmol of L-γ-glutamyl hydroxamate formed per h per mg of protein at 37 °C. The reaction was tested for linearity and dependence on the protein concentration employed.

Immunofluorescence staining of GS

For immunofluorescence, HSC were cultured on glass cover slips with a diameter of 10 mm at a density of 0.1×10^6 cells/cover slip. At the end of culture time, cells were fixed with methanol for 10 min at 4 °C. After rinsing twice with PBS, cells were incubated in PBS containing 0.1 % Triton for 10 min at room temperature. The cells were washed again and incubated for at least 3 h at room temperature with mouse anti-GS antibody (1:20 dilution) in a wet chamber. Then the cells were washed with PBS and the incubation was continued over 1 h with FITC-conjugated anti-(mouse immunglobulin) (1:25 dilution) plus propidium iodide (1:1000 dilution). Control cultures were processed without initial incubation with the specific antibody. Microscopy was performed using a confocal laser scanning system (argon/krypton laser; Leica, Benzheim, Germany). Light of 488 nm wavelength was used for excitation, and images were acquired at 515–545 nm for FITC and 590 nm for propidium iodide.

Northern blot analysis

Total RNA from culture plates of HSC was isolated by using the RNeasy Total RNA Kit (Qiagen). RNA samples (2 μg per lane) were electrophoresed in 0.8 % agarose containing 3 % formaldehyde and then blotted on to Hybond N nylon membranes (Amersham) with 20 × SSC (3 M NaCl/0.3 M sodium citrate). After brief rinsing with water and UV-cross-linking (Hoefer UV-crosslinker 500), the membranes were observed under UV illumination to determine RNA integrity and location of the 28 S and 18 S rRNA bands. The blots were then subjected to prehybridization for 3 h at 43 °C in 50 % deionized formamide in sodium phosphate buffer (0.25 M, pH 7.2) containing 0.25 M NaCl, 1 mM EDTA, 100 mg/ml salmon sperm DNA and 7 % (w/v) SDS. Hybridization was carried out in the same solution with approx. 10^6 c.p.m./ml [α-^{32}P]dCTP-labelled random-primed GS and GAPDH cDNA probes. Membranes were washed three times in 2 × SSC/0.1 % SDS for 10 min, twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/0.1 % SDS and twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/1 % SDS. Blots were then exposed to Kodak X-OMAT AR-5 film at −70 °C with intensifying screens.

Suitably exposed autoradiograms were then analysed by densitometry scanning (PDI, New York, NY, U.S.A.) to determine the absorbances of the mRNAs for GS and GAPDH. Relative GS mRNA levels were determined by standardization to the absorbance of GAPDH mRNA.

Analysis of results

Values are expressed as means ± S.E.M. (n = 3–6). Results of at least three independent experiments are given. For statistical analysis, one-way analysis of variance was used; $P < 0.05$ was considered to be statistically significant.

RESULTS

As shown in Figure 1, in quiescent HSC cultured for 1 day neither α-smooth-muscle actin nor GS protein was detectable by Western blot analysis, and immunocytochemical staining for GS

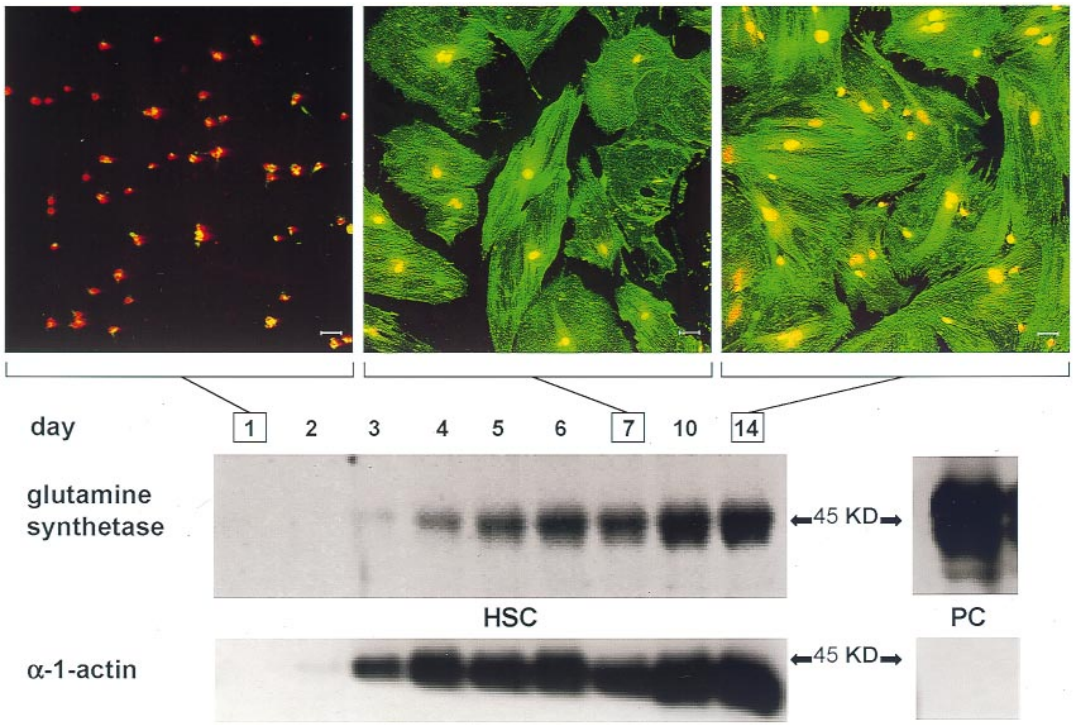

Figure 1 GS and α-smooth-muscle actin expression in HSC and during their transformation into myofibroblast-like cells

Isolated HSC were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 12 mM glucose and 4 mM glutamine for the time periods indicated. Then cells were harvested for Western blot analysis using antibodies raised specifically against GS or α-smooth-muscle actin (α-1-actin) as a parameter of activation. Lysates from parenchymal cells (PC) were used as a positive control. For immunofluorescence, cells were stained using an antibody raised specifically against GS and with propidium iodide for nuclear staining. At day 1, no GS was detectable at the protein level and only nuclear staining was visible by immunofluorescence. After about 3 days of culture, GS became detectable, and levels increased further over the subsequent culture period, as shown by Western blot analysis and immunofluorescence staining. (Scale bar: 60 μm.)

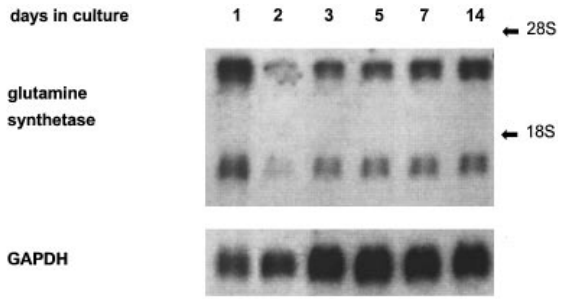

Figure 2 GS mRNA in HSC during culture

Cells were harvested for RNA isolation after the time periods indicated and subjected to Northern blot analysis for GS and GAPDH. The positions of the 28 S (≈ 4.7 kb) and 18 S (≈ 1.9 kb) rRNA bands are depicted.

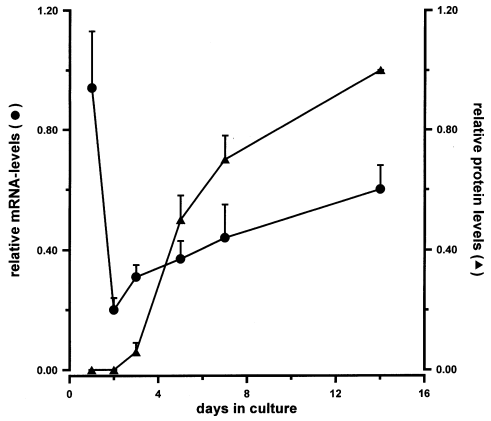

Figure 3 Relative GS mRNA and protein levels in HSC in culture

GS mRNA levels were analysed by determination of density, and relative GS mRNA levels (●) are represented by the ratio GS mRNA/GAPDH mRNA. The GS protein level (▲) measured on day 14 of culture was arbitrarily set to 1.0, and the protein content found at other time points is expressed as a fraction thereof. Results are from three or four independent experiments and are expressed as means ± S.E.M.

and α-smooth-muscle actin (results not shown) was negative. In line with the absence of GS protein from quiescent HSC, no GS activity was detectable in cell lysates. On the other hand, the GS activity in 24 h-cultured liver parenchymal cells was 0.57 ± 0.03 μmol/h per mg of cell protein ($n = 5$). These values are similar to the average GS activity found in separately isolated perivenous and periportal parenchymal cells [17]. When HSC were kept in culture for longer periods, they were transformed into myofibroblast-like cells, as assessed by the expression of α-smooth-muscle actin (Figure 1). In parallel with this, GS was expressed; the appearance of the enzyme in activated HSC was confirmed by Western blot analysis and intense immunocytochemical staining for GS. Whereas GS activity in HSC cultured for 1 day was 0.01 ± 0.00 μmol/h per mg of protein

($n = 4$), it rose to 0.13 ± 0.01 μmol/h per mg ($n = 6$) and 0.14 ± 0.01 μmol/h per mg ($n = 4$) after 7 and 14 days of culture respectively.

Although neither GS protein nor activity was detectable in quiescent HSC, GS mRNA was present in these cells (Figure 2). The pattern of two different mRNA species expressed by HSC fits well with that described in liver tissue from the rat and many other vertebrates [18], and has previously been suggested to be found exclusively in perivenous parenchymal cells [19]. mRNA levels were high during the first day of culture (Figure 3); they declined by 70 % on the second day, but then increased again during the subsequent days of culture, in parallel with GS protein. The relative GS mRNA levels found at day 1 of culture of HSC [0.94 ± 0.1 ($n = 5$)] were comparable with those in 1-day-cultured parenchymal cells [0.79 ± 0.1 ($n = 3$)].

DISCUSSION

The present study shows the *de novo* expression of GS in HSC following their activation in culture, and the appearance of this enzyme may be used as another marker of HSC activation. The absence of GS protein from freshly isolated HSC or cells kept for 1–2 days in culture is in line with previous studies showing the absence of immunostainable GS in non-parenchymal cells of normal rat liver [5]. Surprisingly, despite the absence of GS protein and activity in quiescent HSC, GS mRNA was present. The possibility that the presence of GS mRNA was due to contaminating liver parenchymal cells is unlikely, because such contamination should also result in the presence of GS protein and activity. These, however, were not detectable. Further, no contamination of HSC cultures at day 1 with parenchymal cells was detectable by morphological examination of the cultures. Since parenchymal cells and HSC have almost equal relative GS mRNA levels, the level of contamination with parenchymal cells required to explain the GS mRNA levels found in 1-day-cultured HSC would be more than 50–70 %.

Thus GS mRNA was present in quiescent HSC, but the enzyme protein was not expressed. Such a situation has also been described for parenchymal cells during development: in the developing mouse liver, GS mRNA was expressed before birth, while the protein became detectable 2–3 days after birth [20]. Similar results have been published for the developing rat liver [21]. However, in the developing liver, the level of GS mRNA remains constant, while in cultured HSC a decrease in GS mRNA is found between days 1 and 2, followed by an increase during the subsequent culture period (Figure 3). Thus the present findings suggest that the expression of GS may be regulated in HSC at both the transcriptional and the post-transcriptional levels. In this respect, it is interesting to note that nuclear run-on assays for the GS gene showed marked transcriptional activity in periportal liver parenchymal cells, although the enzyme protein and mRNA are found exclusively in perivenous hepatocytes [22]. These findings were also interpreted to indicate that post-transcriptional events, presumably mRNA degradation, contribute to the heterogeneous expression of GS in liver parenchyma.

The functional implications of the *de novo* expression of GS in HSC during transformation are unknown. However, one may speculate that activated GS-expressing HSC may participate in ammonia detoxification in the injured liver. In human liver cirrhosis [1] and tetrachloromethane-injured rats [8], the hepatic capacity to synthesize glutamine is decreased by more than 80 %, and the number and staining intensity of perivenous GS-containing parenchymal cells (so-called scavenger cells [1,3]) is markedly decreased. Thus liver injury will lower the GS activity in parenchymal cells to values even below those found in the present study in activated HSC, when the activity calculation is expressed on a mg of cell protein basis. HSC make up only 5–8 % of all liver cells, and occupy only 1.4–1.9 % of liver volume in normal liver [11]. This would suggest only a marginal contribution of HSC to total liver GS activity. However, in tetrachloromethane-induced liver injury, the number of pericentral HSC is increased about 7-fold [23]. This suggests an increase in the relative fraction of HSC, and consequently in the HSC-associated GS activity, in cirrhosis or the injured liver.

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