LIVER FIBROSIS

Hepatic stellate cells express synemin, a protein bridging intermediate filaments to focal adhesions

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Background and aims: In the liver, stellate cells play several important (patho)physiological roles. They express a broad but variable spectrum of intermediate filament (IF) proteins. The aim of this study was to investigate the expression and functions of the intermediate filament protein synemin in hepatic stellate cells (HSCs).

Methods: In isolated and cultured rat HSCs, synemin expression was examined by quantitative reverse transcriptase polymerase chain reaction, western blotting, and immunocytochemistry. Protein–protein interaction between synemin and possible binding partners was investigated by co-immunoprecipitation and confocal microscopy.

Results: Expression of synemin was significantly downregulated with increased culture time. In 1-day cultured HSCs, synemin associated with other IF proteins (GFAP, desmin, and vimentin), and with the focal adhesion proteins vinculin and talin, but not with α -actinin or paxillin. Synemin IF and focal adhesion proteins co-localised in long slender processes, but not in the lamellipodia. In human and rat liver tissue, the presence of synemin was investigated by immunohistochemistry. In normal rat and human livers, synemin immunoreactivity was found in HSCs, smooth muscle cells of hepatic arterioles, and nerve bundles in portal tracts, but not in portal fibroblasts. In CCl₄-intoxicated rat livers and in human cirrhotic livers, immunoreactivity for synemin in the parenchymal tissue was decreased. Thus synemin was expressed in quiescent HSCs but not in portal fibroblasts; and synemin expression decreased with HSC activation in vivo during chronic liver damage and with HSC activation in culture.

Conclusions: Synemin forms heteropolymeric filaments with type-III IF proteins and acts as a bridging protein between IFs and a specific type of focal adhesions.

Hepatic stellate cells (HSCs) play a pivotal role in vitamin A metabolism, in the pathogenesis of liver fibrosis and cirrhosis, in the development of portal hypertension^{1,2} and in progression of liver cancer.³ In response to growth factors and cytokines during chronic injury, HSCs change from vitamin A storing quiescent cells to myofibroblast-like cells that are important producers of extracellular matrix (ECM).⁴ Activated HSCs express α -smooth muscle actin (α -SMA) and acquire the ability to proliferate, to synthesise a large variety of ECM molecules, to secrete cytokines and growth factors, and to migrate and contract. This activation process of HSCs is closely reproduced when cells are cultured on plastic dishes.

Recently it has also been shown that integrin mediated interactions with the surrounding ECM have a profound influence on the phenotype and the functional state of HSCs.^{5–7} The binding of ligands such as fibronectin or collagen type I can result in integrin activation and in the recruitment of structural and signalling molecules such as vinculin, paxillin, talin, and focal adhesion kinase that together form complexes called focal adhesions. In turn, these signalling molecules control many aspects of cell phenotype, differentiation, cell survival, and cell cycle progression.^{8,9} Moreover, integrins provide a structural link between the ECM and F-actin through talin, vinculin, α -actinin, or filamin, or combinations of these, enabling tissues to withstand mechanical stress.^{8–11}

Intermediate filament proteins are cytoskeletal proteins that form 10 nm diameter intracellular filaments. Based on sequence homologies, intermediate filament (IF) proteins are subdivided into five distinct types and one miscellaneous

type.¹² Synemin was first described as a protein that co-purified from muscle with desmin and vimentin.¹³ Some investigators rank synemin as a type IV IF protein,¹² whereas others classify it as type VI.¹⁴ It has been reported that synemin is expressed in skeletal muscle, cardiac muscle, intestine, lung, colon, prostate, erythrocytes, astrocytes, and eye cells.^{15–17} Synemin was shown to contain a rod domain of approximately 310 amino acids, characteristic of IF proteins.¹⁸ In man, synemin occurs in two isoforms derived from the same gene by alternative splicing.¹⁷ Human synemin α and β have a very short N-terminal domain of 10 amino acids and a long C-terminal tail domain consisting of 1243 amino acids for the α isoform and 931 amino acids for the β isoform.¹⁷ Synemin β is also known as desmuslin.¹⁹ Synemin cannot form IFs by itself and is always present as a heteropolymeric IF with at least one other major IF protein.^{20–22} In striated muscle cells, these heteropolymeric IFs are located at the periphery of myofibrillar Z lines and link adjacent myofibrils. Synemin containing IFs also extend from the Z lines of the peripheral layer of cellular myofibrils to the costameres located periodically along the inside of the sarcolemma.²¹ The C-terminal domain of synemin binds to actin binding protein α -actinin and the focal adhesion protein vinculin.^{20,21} Therefore, it has been proposed that synemin

Abbreviations: α -SMA, α -smooth muscle actin; DAB, 3, 3'-diaminobenzidine; ECM, extracellular matrix; EGTA, ethylene glycol tetra-acetic acid; GBSS, Gay's balanced salt solution; GFAP, glial fibrillary acidic protein; HEPES, N-2-hydroxypiperazine-N'-2-ethanesulphonic acid; HSC, hepatic stellate cell; IF, intermediate filament; PBS, phosphate buffered saline; QRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulphate

Table 1 Antibodies used in the study

Antibody	Source	Dilution		
		IHC	ICC	Western
<i>Primary rabbit polyclonal antibodies</i>				
Anti-turkey synemin	R Robson	1:400		
Anti-human synemin	Z Li	1:800	1:800	1:1000
Anti-vinculin (H-300) sc-5573	Santa Cruz		1:500	1:1000
<i>Primary mouse monoclonal antibodies</i>				
Anti-porcine desmin clone DE-U-10 (IgG1)	Sigma	1:300	1:500	1:1000
Anti-human vimentin clone VIM3.2 (IgM)	Sigma	1:500	1:500	1:1000
Anti-porcine GFAP (IgG1) clone G-A-5	Sigma	1:2000	1:500	1:1000
Anti- α -smooth muscle actin (IgG2a) clone 1A4	Sigma	1:250		1:1000
Anti- β -actin clone AC-15 (IgG2a)	Sigma			1:2000
Anti-vinculin clone hVIN-1 (IgG1)	Sigma		1:2000	1:1000
Anti-talin clone 8D4 (IgG1)	Sigma		1:250	1:1000
Anti- α -actinin (IgM) clone BM-75.2	Sigma	1:500	1:500	1:1000
Anti-paxillin (IgG1) clone 5H11	Upstate			1:250
Anti-plectin (IgG1) clone 7A8	Sigma			1:200
<i>Secondary antibodies</i>				
Anti-mouse antibody HRP conjugated	Amersham	1:500		1:2000
Anti-mouse antibody AP conjugated	Amersham	1:500		1:2000
Anti-rabbit antibody HRP conjugated	Amersham	1:500		1:2000
Anti-mouse IgG antibody Alexa Flour 488	Molecular Probes		1:200	
Anti-mouse IgG antibody Alexa Flour 633	Molecular Probes		1:200	
Anti-mouse IgG1 antibody Alexa Flour 488	Molecular Probes		1:200	
Anti-mouse IgG2a antibody Alexa Flour 633	Molecular Probes		1:200	
Anti-rabbit IgG antibody Alexa Flour 488	Molecular Probes		1:200	
Anti-rabbit IgG antibody Alexa Flour 633	Molecular Probes		1:200	

AP, alkaline phosphatase; HRP, horseradish peroxidase; ICC, immunocytochemistry; IHC, immunohistochemistry; Western, western blotting.

connects synemin containing heteropolymeric IFs to myofibrils through its interaction with α -actinin, and to costameres through its interaction with vinculin or α -actinin or both.²¹

In the present study, we have investigated whether HSCs express synemin. We found that quiescent rat and human HSCs express two isoforms of this protein. We next studied the regulation of synemin expression and found that it decreases during both in vivo and in vitro activation of HSCs. We found that synemin forms heteropolymeric filaments with type III IF proteins and acts as a cytoskeletal cross-linking protein that connects IFs to focal adhesions inside the cytoplasmic processes of quiescent HSCs.

METHODS

Rat and human liver tissue

Human liver tissue samples for histological studies were collected during liver surgery in the Queen Elisabeth Hospital, Birmingham, UK. Before tissue collection, permission by the local ethics committee and informed patient consent were obtained. For histology, normal human liver tissue and diseased liver tissues from patients with primary biliary cirrhosis and haemochromatosis were studied. Male Wistar rats (250 to 300 g) were also used for histological studies. The experiments involving rats were conducted according to the guidelines on the use of animals for

biomedical research imposed by the Belgian National Fund for Scientific Research, and after having obtained permission of the local ethics committees at the Universities of Brussels (Belgium) and Newcastle upon Tyne (UK). For histology, either normal rats or rats exposed to carbon tetrachloride (CCl₄) were used. The latter were given intraperitoneal injections with 100 μ l/100 g body weight of CCl₄ twice weekly for two, four, or six weeks.²³ CCl₄ was dissolved 1:1 (vol/vol) in pharmaceutical grade paraffin oil. Liver tissues were snap frozen and kept in liquid nitrogen. Upon usage, frozen tissue blocks were trimmed and mounted on aluminium stubs with optical cutting temperature (OCT) compound (Agar Scientific, Stansted, UK).

Immunohistochemistry

Frozen sections (10 μ m thick) were cut and dried. They were fixed in 100% acetone for 10 minutes at -20°C . After blocking with 4% albumin/phosphate buffered saline (PBS), the sections were reacted with primary antibody for one hour at 25°C . After rinsing three times for 10 minutes each, the sections were incubated with peroxidase conjugated secondary antibody for one hour at 25°C . Peroxidase was visualised with 3,3'-diaminobenzidine (DAB)/H₂O₂ enhanced with Ni²⁺ and Co²⁺ ions.^{23, 24} When appropriate, tissue was counterstained with Harris haematoxylin for 30 seconds and

Table 2 Sequence of primers and probe: primers for reverse transcriptase polymerase chain reaction

	Accession numbers	Forward primer	Reverse primer	Length	Cycle
Synemin (α + β)	–	GCAAGACGAGATTGCACAGG	CCATTAAGGTCACAGAACCGC	404	30
Synemin (α)	Dr Z Li, personal communication	CCCACAGACATTGGGATTIAGG	CACCCATCTGAAAGGTAAATGAAGTT	401	30
Synemin (β)	NM_182737	GAAGGATGATACTGACCGGGTC	GTCTGTCAAATCCAGCCTGCT	426	30
GFAP	NM_017009	TGGCCACCACTAATGATGCA	GACTCCTTAATGACCTCGCCAT	538	32
Desmin	NM_022531	AGACTTGACTCAGGCAGCCAAT	CGGAAGTTGAGAGCAGAGAAGG	384	25
Vimentin	NM_031140	CGCCAGCAGTATGAAAGTGTG	AGTGGGTGTCAACCAGAGGAA	496	25
18srRNA	AY248756	GAGGTGAAATCTTGGACCGG	TGGCTGAACGCCACTTGTG	513	20

Table 3 Sequence of primers and probe: primers and probes for quantitative reverse transcriptase polymerase chain reaction			
	Forward primer	Probe	Reverse primer
Synemin ($\alpha+\beta$)	CGGATGCGCGAGGAGTAC	TCACTCTCTGCCGCTCCT	GGTAAGGGCATCCTTCTCATCCT
Synemin (α)	GCAGTAGCACCATGTACACA	TTTGGGCCCCAGTGCAA	GTCACCTACACCATCTGAAAGGT
Synemin (β)	CCTACGGAGGACCCACCT	CACCATCTCCACCACCGG	GGATGGCTCTCGTGCTACTTA

mounted in Aquamount (BDH, Poole, UK). Sources and dilutions of the antibodies are summarised in table 1.

Isolation and culturing of rat HSCs

Male Wistar rats (350 to 400 g) were used to isolate and purify HSCs. The HSC isolation method was a modification of the

previously described method for rat HSCs.^{25 26} Briefly, the liver was perfused for 10 minutes with SC-1 solution consisting of 8000 mg/l NaCl, 400 mg/l KCl, 88.17 mg/l, NaH₂PO₄ H₂O, 120.45 mg/l Na₂HPO₄, 2380 mg/l HEPES, 350 mg/l NaHCO₃, 190 mg/l EGTA, 900 mg/l glucose, pH 7.3, followed by digestion at 37°C for seven minutes with 0.04% pronase E (Merck,

Figure 1 Analysis of synemin expression at the RNA level by rat hepatic stellate cells (HSCs) in culture. (A) Expression of synemin $\alpha+\beta$, synemin α , synemin β , GFAP, desmin, vimentin, and 18sRNA in cultured rat HSCs at day 1, 3, 7, 14, or 21 using reverse transcriptase polymerase chain reaction. This indicates that expression of GFAP, synemin $\alpha+\beta$, synemin α , and synemin β was decreased with time in culture, while expression of vimentin was upregulated. Expression of desmin was transiently upregulated during culture. (B–H) Quantification of synemin RNA expression in cultured HSCs by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). (B–D) QRT-PCR analysis (comparative Ct method) and (E–H) amplification plots of 18s RNA (internal control) synemin $\alpha+\beta$, α , and β . Expression of synemin $\alpha+\beta$, synemin α , and synemin β was significantly downregulated during the culture induced activation. Compared with the RNA expression of total synemin in 1-day cultured HSCs, its expression in 3, 7, 14, or 21 days cultured HSCs was reduced to 41%, 7%, 2%, and 1.6%, respectively (B and F). RNA expression of synemin α in 3, 7, 14, or 21 day cultured HSCs was reduced to 47%, 5.1%, 2.7%, and 1.4% of that of 1-day cultured HSCs, respectively (C and G). RNA expression of synemin β in 3, 7, 14, or 21 day cultured HSCs was reduced to 40%, 9%, 3.7%, and 1.8% of that of 1-day cultured HSCs, respectively (D and H).

Figure 2 Analysis of synemin protein expression by rat hepatic stellate cells (HSCs) in culture. (A) Western blotting for synemin isoform in cultured HSCs (day 1), skeletal muscle, stomach, and brain. Synemin reactive bands were detected at an apparent molecular mass of 200 kDa (synemin α) and 180 kDa (synemin β). Synemin β was expressed by cultured HSCs, skeletal muscle, smooth muscle, and brain. Synemin α was also expressed by cultured HSCs and in smooth muscle, but not skeletal muscle or brain. The expression ratio of synemin α /synemin β was 21% (HSCs), 0% (skeletal muscle), 18% (smooth muscle), and 0% (brain). Another band was detected at the molecular weight of 170 kDa (degradation product of synemin α) in smooth muscle. (B) Western blotting for synemin, GFAP, desmin, vimentin, α -SMA, and β -actin by using cultured HSCs. Cultured HSCs expressed synemin, GFAP, desmin and vimentin. During time in culture, expression of GFAP, synemin α and β was significantly downregulated, while expression of vimentin and α -SMA was upregulated. Desmin was transiently upregulated during culture. (C, D) Western blotting analysis for synemin expression by densitometry. Expression of synemin α and β was significantly downregulated during time in culture. Protein expression of synemin α in 3, 7, 14, or 21 day cultured HSCs was reduced to 63%, 14%, 0%, and 0%, respectively, of that of day 1 cultured HSCs. Protein expression of synemin β in 3, 7, 14, or 21 day cultured HSCs was reduced to 59%, 21%, 0%, and 0%, respectively, of that of day 1 cultured HSCs.

Darmstadt, Germany) and for seven minutes with 0.02% collagenase (Roche Diagnostics Corporation, Mannheim, Germany) dissolved in SC-2 solution consisting of 8000 mg/l NaCl, 400 mg/l KCl, 88.17 mg/l NaH_2PO_4 , H_2O , 120.45 mg/l Na_2HPO_4 , 2380 mg/l HEPES, 350 mg/l NaHCO_3 , and 560 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.3. The digested liver was excised, cut into small pieces, and incubated at 37°C for 20 minutes in SC-2 solution containing 0.03% collagenase, 0.04% pronase E, and 0.001% deoxyribonuclease (Roche Diagnostics Corporation). The resulting suspension was filtered through a 100 μm nylon mesh and centrifuged on an 8% Nycodenz cushion (Myegaard, Oslo, Norway), which produced an HSC enriched fraction in the upper whitish layer. After isolation, cells were suspended in Dulbecco's modified Eagle's medium (Cambrex Bio Science, Verviers, Belgium) with 10% fetal calf serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, plated on plastic dishes (Becton Dickinson, Le Pont De Claix, France), and cultured at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Culture medium was renewed 20 hours after plating. The purity of HSCs in the culture was greater than 90% assessed by phase contrast microscopy. Cultured HSCs were trypsinised and replated at day 7.

RNA preparation and reverse transcriptase polymerase chain reaction

Total RNA was extracted from cultured HSCs, rat skeletal muscle, stomach, and brain by using TRIZOL Reagent

(Invitrogen, Merelbeke, Belgium). The purification procedures were carried out according to the manufacturer's protocols. The concentration, purity, and amount of total RNA were determined by spectrophotometry. By these standards, all the RNA samples used for the assays were of high quality and purity ($\text{Abs}_{260}/\text{Abs}_{280} > 1.7$). Total RNA was reverse transcribed using a high capacity cDNA archive kit (Applied Biosystems, Foster City, California, USA). Reverse transcription was undertaken at 25°C for 10 minutes, followed by 37°C for 120 minutes. Polymerase chain reaction (PCR) was carried out using a platinum Taq DNA polymerase kit (Invitrogen) with a total amount of 50 ng cDNA template. The conditions for reverse transcriptase polymerase chain reaction (RT-PCR) were as follows: five minutes at 95°C and then 20 to 30 cycles of amplification (30 seconds at 95°C, 30 seconds at 60°C, one minute at 72°C) and extension for 10 minutes at 72°C. Primers were designed by Primer Express. Primers and the number of PCR cycles used are summarised in table 2. Amplification products were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. The identity of the amplified products was confirmed by automatic sequencing (Applied Biosystems) and by BLAST search in the NCBI database.

Quantitative reverse transcriptase polymerase chain reaction

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was undertaken using the ABI 7700 SDS

Figure 3 Association of synemin with other intermediate filament (IF) proteins. (A) Co-immunoprecipitation by anti-synemin antibody. Cell lysates from day 1 cultured hepatic stellate cells (HSCs) were immunoprecipitated (IP) by a polyclonal anti-synemin antibody followed by western blotting with anti-desmin, anti-GFAP, anti-vimentin, anti-synemin, and non-immune immunoglobulin (negative control), as described in Methods. These results indicate that synemin associated with GFAP, desmin, and vimentin. Negative control: negative control experiment. (B) Co-localisation of synemin and other IF proteins in 1 day cultured HSCs by double fluorescent staining. HSCs cultured for one day were stained with a polyclonal anti-synemin antibody (SYN) (left column) and a monoclonal antibody for GFAP, desmin (DES), or vimentin (VIM) (middle column). Merged images are shown in the right column. These data indicate that synemin co-localised with other IF proteins such as GFAP, desmin, and vimentin. GFAP and desmin overlapped strongly with synemin, while vimentin partially overlapped with synemin. Bar = 10 μ m. GFAP, glial fibrillary acidic protein.

(sequence detection system) reaction and detection system (Applied Biosystems). Primers and probes for synemin α + β , synemin α , and synemin β were made by Assays-by-Design Gene Expression Product system (Applied Biosystems). Predeveloped TaqMan assay reagent for 18S ribosomal RNA (18S rRNA) (Applied Biosystems) was used as an internal control. We mixed TaqMan universal PCR master mix, cDNA template (50 ng per reaction), and Assays-by-Design gene expression product for synemin or predeveloped TaqMan assay reagent for 18S rRNA, including two unlabelled PCR primers and a FAM dye labelled TaqMan MGB probe. The conditions of QRT-PCR were as follows: two minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of amplification (denaturation for 15 seconds at 95°C and extension for one minute at 60°C). Primers and probes for QRT-PCR are summarised in table 3. For analysis according to the Delta-Delta threshold (Ct) method,²⁷ each Ct value was first normalised to the respective 18S rRNA Ct value of the

sample, and afterwards to the control. The fold induction was calculated from these Ct values.

Western blotting

Cells were homogenised in sample buffer (62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS, 1 mM NaF, 1 mM Na₃VO₄, and 10% glycerol), and boiled for 10 minutes. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, Illinois, USA). Samples were prepared for loading by adding 5% β -mercaptoethanol and 0.005% bromophenol blue. Samples were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane (Milipore, Bedford, Massachusetts, USA). After blocking with 5% skimmed milk, 0.1% Tween 20 in Tris-buffered saline at 4°C overnight, the membranes were reacted with primary antibodies for two hours at 25°C. The membrane was then incubated with secondary antibody for one hour at 25°C. The antigens were

Figure 4 Association of synemin with focal adhesion proteins. (A) Western blotting for focal adhesion proteins in cultured hepatic stellate cells (HSCs). Cultured HSCs expressed vinculin, α -actinin, talin, paxillin, and plectin. During culture, expression of α -actinin, talin, paxillin, and plectin were significantly upregulated, while that of vinculin remained rather stable. (B) Western blotting for vinculin isoforms in HSCs and skeletal muscle, stomach, and brain. Vinculin (VCL) reactive bands were detected at 150 kDa (meta-vinculin) and 130 kDa (vinculin) in skeletal muscle and stomach, while only one band was detectable at 130 kDa (vinculin) in cultured HSCs and brain. (C) Co-immunoprecipitation with anti-synemin antibody. Cell lysates from 1 day cultured HSCs were immunoprecipitated (IP) with a polyclonal anti-synemin antibody followed by western blotting with anti-vinculin, anti- α -actinin, anti-talin, and anti-paxillin antibodies. One reactive band for vinculin and two reactive bands for talin (talin at 225 kDa and its proteolytic fragment at 190 kDa) were found. No reactive bands for α -actinin and paxillin were found.

detected by enhanced chemiluminescence using ECL substrate (Amersham, Roosdaal, Netherlands). The antibodies used for western blotting are summarised in table 1.

Confocal microscopy

In order to identify co-localisation of synemin with other proteins, double and triple fluorochrome labelling was carried out in 1-day cultured HSCs. Cultured HSCs grown on glass cover slips were fixed for 10 minutes in periodate-lysine-paraformaldehyde (PLP) at 25°C and extracted in 0.1% Triton X-100 in PBS for 10 minutes. Following three washes with PBS, cells were preincubated with 1% albumin/PBS for 30 minutes, and incubated with polyclonal anti-synemin antibody and monoclonal anti-desmin, anti-GFAP, anti-vimentin, anti-vinculin, or anti-talin antibody for one hour. Following three washes with PBS, the cells were incubated with the appropriate fluorochrome conjugated secondary antibodies for one hour (see table 1). The cells were mounted in fluorescent mounting medium (Dako, Glostrup, Denmark), and then observed using the Leica TCS SP confocal laser scanning microscope (Heidelberg, Germany) with a 63 $\times$ water immersion objective lens. Images were made with the Leica-CSLM 1.6.587 TCS-NT software, and the digital CSLM images obtained were transferred to Adobe PhotoShop 5.5 software for colour channel analysis and figure assembly.

Immunoprecipitation

Cells on 10 cm culture dishes were rinsed three times with PBS and lysed in 0.5 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% SDS, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4 , and protease inhibitor mixture (Roche Diagnostics)). Insoluble material was removed by centrifugation at 4°C. After total cell protein in the lysates had been determined by the BCA protein assay kit, synemin antibody was added to the cell lysates and incubated for two hours at 4°C, followed by precipitation on protein-A-agarose

beads (Roche Diagnostics) overnight. The immunoprecipitated proteins were washed with lysis buffer and then denatured with sample buffer (62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS, 1 mM NaF, 1 mM Na_3VO_4 , 10% glycerol, 5% β -mercaptoethanol). Samples were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane (Milipore). After blocking with 5% skimmed milk, 0.1% Tween 20 in Tris buffered saline at 4°C overnight, the membranes were reacted with primary antibodies for two hours at 25°C. The membrane was then incubated with secondary antibody for one hour at 25°C. The antigens were detected by enhanced chemiluminescence using ECL substrate (Amersham, Roosdaal, Netherlands). The antibodies used for western blotting are summarised in table 1.

Statistics

Unless otherwise stated, the experiments described here were repeated on liver tissue from three animals per group or on three independent cell isolates. The data are expressed as mean (SD). The statistical significance of the difference between two sample groups was calculated by the Mann-Whitney test. The difference was considered significant at $p < 0.05$.

RESULTS

Expression of synemin mRNA is downregulated in cultured rat HSCs

We investigated synemin mRNA steady state levels in rat HSCs at day 1, 3, 7, 14, or 21 in culture and found that total synemin mRNA levels, as well as those of synemin α and β , were clearly detectable at day 1 in culture, but declined with culture time (fig 1A). In order to quantify the change of steady state levels during culture, we carried out QRT-PCR. When compared with the mRNA levels of synemin α and β of cells at 1 day in culture, the levels in 3, 7, 14, or 21 day cultured HSCs were reduced to 41 (11)%, 7.5 (4.9)%, 2.7

Figure 5 Co-localisation of synemin and focal adhesion proteins in hepatic stellate cells (HSCs) by double immunofluorescence staining. HSCs cultured for 1 day were stained with a polyclonal anti-synemin antibody (SYN) (B, G) and a monoclonal antibody to vinculin (VCL) (A) and talin (TAL) (F). Cells were viewed by confocal microscopy. Vinculin and talin were present in cytoplasmic processes as well as in lamellipodia (A, F). Synemin filaments (SYN) extended from the perinuclear area into the peripheral cytoplasm of slender processes (B). Merged images are shown in (C) and (H). Co-localisation of synemin (SYN) and vinculin (VCL) was located in the processes (D, arrows). On the other hand, in the areas where the cell body was spreading and process formation was disappearing (arrowheads), co-localisation was diminished compared with peripheral processes (E). Co-localisation of talin and synemin is also present in the processes (I, arrows), but not in the lamellipodia (J, arrowheads). Bar (A–C and F–H), 10 μ m; (D, E, I, and J), 5 μ m.

(1.2)%, and 1.6 (0.7)%, respectively (fig 1, panels B and F). Steady state levels of synemin α mRNA in 3, 7, 14, or 21 day cultured HSCs were reduced to 47 (11)%, 5.1 (2.6)%, 2.7 (1.0)%, and 1.4 (0.7)% compared with 1 day cultured HSCs (fig 1, panels C and G). Levels of synemin β mRNA in 3, 7, 14, or 21 day cultured HSCs were reduced to 40 (7)%, 9.0 (5.9)%, 3.7 (2.0)%, and 1.8 (0.6)% of those of 1 day cultured HSCs (fig 1, panels D and H). From these results, we conclude that RNA steady state levels of both synemin α and

synemin β decreased rapidly during culture induced HSC activation.

Synemin protein steady state level is downregulated in cultured rat HSCs

We investigated synemin protein steady state levels by western blotting. One-day cultured HSCs contained one major and one minor immunoreactive band with apparent molecular mass of 180 and 200 kDa, representing the

Figure 6 Co-localisation of β -actin (BAT) and vinculin (VCL) or synemin (SYN) in hepatic stellate cells (HSCs) by double immunofluorescence staining. HSCs cultured for one day were stained with a monoclonal anti- β -actin antibody (IgG2) (A, F), a monoclonal antibody for vinculin (IgG1) (B), and a polyclonal antibody for synemin (G). Cells were viewed by confocal microscopy. β -Actin filaments were localised in the cell body of HSC as well as in the peripheral processes (A, F). Merged images are shown in (C) and (H). Co-localisation of β -actin and vinculin was found in the lamellipodia (D, arrowheads) as well as in the processes (E, arrows). β -Actin filaments were overlapping with synemin in the slender processes of HSCs (I, arrows), but not in the areas where processes had disappeared (J, arrowheads). Bar (A–C and F–H), 10 μ m; (D, E, I, and J), 5 μ m.

β isoform and α isoform, respectively (fig 2A).²⁸ The ratios of synemin α /synemin β in 1-day cultured HSCs, skeletal muscle, stomach, and brain were 21 (3)%, 0%, 18 (10)%, and 0%, respectively. Hence, the pattern of synemin isoforms in HSCs is comparable to that of smooth muscle. Next, we assessed synemin protein during culture by western blotting and densitometry (fig 2, panels B–D). Protein levels of synemin α in day 3, 7, 14, or 21 cultured HSCs were reduced to 63 (23)%, 14 (1.7)%, 0%, and 0% of those of 1-day cultured HSCs (fig 2C). Levels of synemin β in 3, 7, 14, or 21 day

cultured HSCs were reduced to 59 (9)%, 21 (13.8)%, 0%, and 0% of those of 1-day cultured HSCs (fig 2D).

Synemin associates with GFAP, desmin, and vimentin

We used co-immunoprecipitation and double labelling immunofluorescence in conjunction with confocal microscopy to investigate interactions between synemin and other IF proteins in 1-day cultured HSCs. We found that synemin co-immunoprecipitates with GFAP, desmin, and vimentin

Figure 7 Co-localisation of β -actin, synemin and vinculin in hepatic stellate cells (HSCs) by triple immunofluorescence staining. HSCs cultured for one day were stained with a monoclonal anti- β -actin antibody (IgG2) (A), a polyclonal anti-synemin antibody (SYN) (B), and a monoclonal antibody for vinculin (IgG1) (VCL) (C). Cells were viewed by confocal microscopy. Merged images are shown in (D). Overlapping staining of β -actin, synemin, and vinculin (white colour) was present in the slender process (E). Arrows indicate the co-localisation of β -actin, synemin, and vinculin. Bar (A–D), 10 μ m.

(fig 3A). Intracellular co-localisation was confirmed by confocal microscopy (fig 3B).

Synemin associates with focal adhesion proteins, vinculin and talin

First, we investigated whether α -actinin and the focal adhesion proteins vinculin, paxillin, talin, and plectin were

present in HSCs. Western blotting confirmed the presence of α -actinin, vinculin, talin, and paxillin (fig 4A). Plectin was barely present in 1-day cultured cells, but became apparent as from day 3 and increased steadily thereafter. Next, we investigated which isoform of vinculin was present in HSCs by western blotting; one vinculin immunoreactive band of 130 kDa was found in cultured HSCs and brain tissue,

Figure 8 Immunohistochemical staining of normal rat liver for synemin (A), desmin (B), and glial fibrillary acidic protein (GFAP) (C). Synemin immunoreactivity was observed in perisinusoidal cells with several cytoplasmic processes (arrows) and occasional lipid droplets (asterisk) (A). A subpopulation of hepatic stellate cells (HSCs) was strongly immunoreactive for desmin (arrows) (B). Antibodies to GFAP stained numerous perisinusoidal HSCs. (C, arrows). No immunoreactivity was observed in control sections incubated with 1/800 normal rabbit serum (D). Magnification of all panels, $\times 370$.

whereas two immunoreactive bands of 130 kDa and 150 kDa were found in skeletal muscle and stomach tissue, indicating that vinculin but not meta-vinculin was expressed by HSCs (fig 4B). During HSC activation, expression of α -actinin and paxillin were upregulated. Talin was transiently upregulated, whereas expression of vinculin was roughly constant (fig 4A). In addition, we investigated the interactions between synemin and α -actinin, or synemin and focal adhesion proteins, in cultured HSCs by co-immunoprecipitation and double labelling immunofluorescence. Co-immunoprecipitation experiments showed that synemin associated with vinculin and talin, but not with α -actinin or paxillin (fig 4C). In order to identify the subcellular location of co-localisation of synemin filaments and focal adhesion proteins, we carried out confocal microscopy. Double immunofluorescence (fig 5, panels A–C) showed that synemin filaments co-localised with vinculin present in the cytoplasmic processes (fig 5, panels C and D), but not in the lamellipodia of HSCs (fig 5, panels C and E). In addition, the longest HSC cellular processes showed more co-localisation of synemin with focal adhesion proteins than did short processes or the cell bodies (fig 5E). Similarly, synemin filaments co-localised with talin (fig 5, panels F, G, and H) in the cytoplasmic processes of HSCs (fig 5I) but not in the lamellipodia of HSCs (fig 5J).

Synemin interacts with β -actin filaments through synemin associated focal adhesions

In order to investigate the co-localisation of β -actin filaments and focal adhesions, we undertook double immunostaining

of β -actin and vinculin (fig 6, panels A–C). This experiment showed that β -actin filaments co-localised with vinculin in the cell bodies of HSCs (fig 6, panels C and D) as well as in the cytoplasmic processes (fig 6, panels C and E). Next, we investigated the co-localisation of β -actin and synemin (fig 6, panels F–H) and found that β -actin filaments co-localised with synemin in some of the cytoplasmic processes (fig 6, panels H and I) but rarely in the cell bodies of HSCs (fig 6, panels H and J). To confirm the co-localisation of synemin, vinculin, and β -actin, we used triple fluorescent immunostaining and confocal microscopy (fig 7, panels A–E). In the cytoplasmic processes, we found areas where synemin, β -actin, and vinculin staining overlapped (fig 7E).

Presence of synemin in normal rat liver

Synemin immunostaining was detected in perisinusoidal cells carrying cytoplasmic processes and containing occasional lipid droplets (fig 8A). The synemin staining co-localised with desmin or GFAP (fig 8, panels B and C), implying that within the hepatic lobules synemin immunoreactive cells are HSCs.

In portal tracts, synemin was present in some axons of nerve bundles and in smooth muscle cells of hepatic arterioles (data not shown). Portal fibroblasts were desmin positive but synemin negative.

Expression of synemin by HSCs is downregulated in CCl₄ mediated liver damage

We exposed rat livers to CCl₄ for two, four, or six weeks and stained the livers for synemin (fig 9). Synemin

Figure 9 Comparison of the immunohistochemistry for synemin in pericentral areas of normal rat livers (A) and livers exposed to CCl_4 for two weeks (B), four weeks (C), and six weeks (D). Synemin immunoreactivity in HSCs was observed in normal livers and in livers exposed to CCl_4 for two weeks (arrows). At four weeks, few HSCs remained positive for synemin, and at six weeks synemin immunoreactivity was absent. Magnification, $\times 220$.

immunoreactivity in HSCs was observed in normal livers and in livers exposed to CCl_4 for two weeks (fig 9, panels A and B) but by four weeks, few HSCs remained positive (fig 9C), and at 6 weeks synemin immunoreactivity was completely absent from HSCs (fig 9D).

Presence of synemin in normal and diseased human liver

In normal human liver tissue, synemin immunoreactivity was present in perisinusoidal cells with cytoplasmic processes (fig 10D). It has been established previously that human HSCs are vimentin immunoreactive.¹ Double immunofluorescence experiments and confocal microscopy were used to confirm that the synemin immunoreactive cells were HSCs. Synemin immunoreactivity clearly overlapped with that of vimentin (fig 10, panels A, B, and C). This observation, together with the typical perisinusoidal location, led us to conclude that synemin immunoreactive cells were HSCs. Portal fibroblasts located in the periportal connective tissue were negative. We also detected synemin immunoreactivity in smooth muscle cells of hepatic arterioles and in some axons of nerve branches.

In addition to normal human liver tissue, we examined diseased liver tissues from patients with chronic inflammatory liver disease and cirrhosis caused by either primary biliary cirrhosis or haemochromatosis. We found no immunoreactive cells in the parenchymal nodules of any of the affected livers, only a few synemin reactive HSCs located near the connective tissue septa (fig 10E). In the connective tissue septa of these diseased livers, we found synemin positive arterioles and nerve bundles but no positive (myo)fibroblasts. These observations indicate that expression

of synemin decreases in stellate cells of human livers with cirrhosis.

DISCUSSION

This is the first detailed account of synemin expression in the liver. It shows that synemin is expressed by quiescent HSCs. Synemin expression is lost during activation, suggesting that this protein is a possible factor in the maintenance of the characteristic morphology of quiescent HSCs.

We report a clear molecular interaction between synemin and other IF proteins expressed by HSCs. We used co-immunoprecipitation and confocal microscopy to show that synemin molecules bind to desmin, vimentin, and GFAP molecules, thereby forming heteropolymeric IFs. Our data confirm and extend previous data obtained by blot overlay experiments^{20–28} and two-hybrid screening.²⁰ These results led us to investigate the presence and composition of focal adhesion in HSCs. We show that quiescent and activated rat HSCs in culture contain bona fide focal adhesions. Confocal microscopy demonstrated vinculin and talin within spot-like or streak-like structures near the cell membrane. Western blotting confirmed that HSCs contain the essential molecular constituents of focal adhesions—that is, vinculin, α -actinin, talin, and paxillin. Activated HSCs also contain plectin. Up to now, only the presence of vinculin has been reported.²⁹ In the future it will be important to determine which integrins are involved in HSC focal adhesions. Quiescent rat HSCs express $\alpha 5 \beta 1$ integrin³⁰; activated HSCs express $\alpha 2 \beta 1$, $\alpha 5 \beta 1$, and $\alpha v \beta 3$.^{30–32} Activated human HSCs express $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, $\alpha 3 \beta 1$, $\alpha 6 \beta 1$, $\alpha v \beta 1$, and $\alpha v \beta 3$.^{32–34} Whether this overview is complete, and which integrin molecules associate with talin or paxillin to form bona fide focal adhesions, remains to be established.

A major finding of our study is the demonstration by co-immunoprecipitation and confocal microscopy of the molecular interaction between synemin and the focal adhesion proteins vinculin and talin, but not α -actinin or paxillin. Previous studies using yeast two-hybrid systems have indicated the binding potential of the α isoform of synemin for vinculin, α -dystrobrevin, and α -actinin.^{19–21} Our study is the first direct proof that these molecular interactions occur. In general, focal adhesions act as attachment plaques at the cell surface. At the cytoplasmic face of the focal adhesion, cytoskeletal filaments bind to integrins through a series of linker proteins including talin, vinculin, filamin, or α -actinin (fig 11, panels A and B).^{10–11} At the extracellular face, integrin molecules bind to extracellular matrix components. Classically, only actin containing microfilaments are thought to bind to the cytoplasmic face of focal adhesions (fig 11A). Recently, focal adhesions were found to interact with microtubules and IFs as well.^{35–38} In human microvascular endothelial cells, vimentin IFs interact with the vimentin associated matrix adhesion through association with plectin (fig 11C).³⁵ This type of adhesion possesses many of the characteristics of classical focal contacts. These constructs are enriched in vinculin and associate with the actin filament system. They are found predominantly at the periphery of endothelial cells.³⁵ We now propose a second variant focal adhesion in which synemin containing IFs interact with vinculin and talin (fig 11D). We name this variant synemin associated focal adhesion. To investigate whether plectin-containing focal adhesions might be present in HSCs as well, we looked for the presence of plectin by western blotting. In quiescent stellate cells there was almost no plectin. Contrary to synemin, which is downregulated during activation, plectin is upregulated. We hypothesize that during activation of stellate cells there might be a shift from synemin containing to plectin containing focal adhesions.

By undertaking triple immunofluorescence and confocal microscopy, we have been able to demonstrate the

Figure 10 Immunohistochemical analysis of synemin in normal and diseased human livers. (A–C): Double immunostaining of synemin and vimentin. Tissues were viewed by confocal microscopy. In intralobular areas, synemin positive cells (SYN) (A) and vimentin positive cells (VIM) (B) were found. Synemin labelling overlapped with that of vimentin (C). Arrows indicate a synemin and vimentin double positive cell. Inset: high magnification of a synemin and vimentin double positive star shaped cell. (D) Synemin reactivity in normal liver. In intralobular areas, numerous perisinusoidal cells were synemin positive. (E) Synemin staining in cirrhotic liver of primary biliary cirrhosis. Except for occasional positive cells close to fibrotic septum (arrow), most stellate cells have lost synemin immunoreactivity. Synemin positive cells in diseased cirrhotic livers at high magnification. Around connective tissue septa, synemin positive HSCs are present (arrows). CT, connective tissue; HP, hepatic parenchymal region. Magnification (A–D), $\times 200$; (E), $\times 400$.

co-localisation of vinculin, synemin, and actin at spot-like structures in the cell membrane. This observation makes it likely that both synemin containing IFs and actin filaments dock onto vinculin in these synemin associated focal adhesions. Moreover, these variant focal adhesions are mainly present in long slender cytoplasmic processes of quiescent HSCs, but not in the much broader lamellipodia, suggesting that synemin associated focal adhesions are important in the formation and maintenance of characteristic quiescent HSC morphology. The processes sprout from the cell body. They project secondary branches that embrace the sinusoidal capillaries³⁹ and contain spike-like protrusions. When HSCs undergo activation, they downregulate the expression of synemin and as a result lose the facility of forming synemin associated focal adhesions. The loss of these variant focal adhesions may contribute to the disappearance of the slender cytoplasmic processes in favour of the development of much broader lamellipodia.

Synemin, lamin A/C, and peripherin are the IF genes that give rise to more than one mRNA transcript. In man, there are two synemin transcripts.^{17–28} The α -synemin transcript of

7.5 kb gives rise to a larger translation product; the β -synemin transcript of 6.5 kb to a smaller translation product. The α -synemin transcript arises when intron IV of the pre-mRNA is not spliced out and hence acts as an additional coding sequence. In mouse, three transcripts of 7.8 (synH), 6.9 (synM), and 4.5 kb (synL) were found.⁴⁰ SynL mRNA arises as a result of splicing out exon IV of the pre-mRNA. In rat tissues, we found two isoforms that we designated synemin- α and synemin- β . These two isoforms are the homologues of α -synemin and synH and of β -synemin and synM in man and mouse. In human skeletal muscle, only the β isoform of synemin occurs. This is also the case in mouse and rat skeletal muscle. In human smooth muscle cells, both isoforms occur in roughly equal quantities, which is also the case in smooth muscle cells of mouse bladder but not in intestinal or respiratory smooth muscle cells, which express the β isoform predominantly. Rat HSCs and stomach smooth muscle cells express approximately five times more β isoform than α isoform. Taking all these observations together, the expression pattern of synemin isoforms appears to be species and cell type dependent, and the expression of 15–20%

Figure 11 Proposed molecular interaction between cytoskeletal filaments and some cytoskeletal associated proteins at the focal adhesion sites. (A, B) Connection between F-actin and focal adhesions. Intracellular domains of β -integrin subunits connect with actin filaments through a series of linker proteins including talin (TAL) and vinculin (VCL) (A) or α -actinin (α -ACT) (B).^{10,11} (C) Connection between vimentin intermediate filament (IF) and vimentin associated focal adhesion. It has been proposed that in endothelial cells vimentin IFs interact with the vimentin associated adhesion via an association with plectin.⁴² This site of adhesion possesses many of the characteristics of focal contact in that it is enriched in vinculin (VCL), associates with actin filament system, and is found predominantly at the periphery of endothelial cells.⁴² The protein that links vinculin to integrin may be α -actinin, whereas the identity of the protein that links plectin to integrins has yet to be determined. (D) Connection between synemin containing IFs and synemin associated focal adhesions. From our results, both synemin containing IFs and actin filaments dock onto vinculin in these synemin associated focal adhesions. In addition, integrins may be involved in this interaction and provide a structural link between the ECM and synemin containing IFs.

α isoform by HSCs suggests that these cells are phenotypically close to intestinal and respiratory smooth muscle cells. There is, however, also a difference between intestinal smooth muscle cells and HSCs. The former express vinculin as well as its splice variant metavinculin,⁴¹ whereas the latter express vinculin but not metavinculin.

In addition to providing new insights into the regulation of HSC differentiation, our study suggests that synemin is a selective marker of quiescent human HSCs, thereby providing a new phenotypic stain to define this subset of mesenchymal liver cells. Neither periportal nor pericentral (myo)fibroblasts contain immunoreactive synemin, making this the first IF protein that is selectively expressed by the vast majority of human HSCs. The expression of synemin by subpopulations of neurones and by arterial smooth muscle cells does not impede the interpretation of the staining patterns because nerve bundles and arterioles have distinct morphological

characteristics and are largely restricted to portal triads. Other marker proteins for quiescent human HSCs—such as cellular retinol binding protein (CRBP),⁴² cytoglobulin/STAP,⁴³ vinculin,^{29,44} or neurotrophins and neurotrophin receptors⁴⁵—do not distinguish between HSCs and periportal fibroblasts, are technically not always easy to carry out,^{29,42} or do not stain the whole population of HSCs. Synemin can be conveniently immunostained on acetone, acetone/ethanol, or PLP fixed sections.

The expression of synemin is clearly downregulated when HSCs acquire the activated phenotype. This pattern of downregulation is similar to the pattern for GFAP that we observed in rat HSCs.²⁴ In all cirrhotic livers studied we found a marked reduction or complete absence of synemin positive cells in the hepatic parenchyma.

Conclusion

Human and rat HSCs express β -synemin and to a lesser extent α -synemin. Their expression is downregulated during in vivo and in vitro HSC activation. Synemin forms heteropolymeric filaments with the type III IF proteins. It also anchors these filaments to focal adhesions through binding to vinculin and talin, thereby producing a variant form of focal adhesion. These synemin associated focal adhesions are abundant in the long slender processes of quiescent HSCs.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1262

The computed tomography scan showed free intraperitoneal gas anterior to the liver. The macroscopic section of the sigmoid colon showed a perforation and adjacent linear ulceration. The histopathologist confirmed there were no microscopic features of inflammatory bowel disease. The diagnosis was non-steroidal anti-inflammatory drug (NSAID) induced distal colonic perforation and ulceration.

NSAIDs can affect the entire gastrointestinal tract. In the normal colon, NSAIDs can cause ulceration, complicated by perforation or haemorrhage, and colitis. In pre-existing colonic disease, NSAIDs may exacerbate diverticulitis and inflammatory bowel disease. Where there is no pre-existing disease, lower gastrointestinal perforation due to NSAIDs is relatively unreported. Associated risk factors for NSAID related gastrointestinal damage include: old age, previous peptic ulceration, perioperative use, smoking, and concomitant drug use (for example, aspirin, anticoagulants, and corticosteroids). There are no data that steroids alone cause colonic bleeding.

This case illustrates that NSAIDs can cause catastrophic lower gastrointestinal complications in a previously healthy gastrointestinal tract, especially in high risk circumstances.

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