Short Communication

Role of the Ets-1 Transcription Factor during Activation of Rat Hepatic Stellate Cells in Culture

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During liver tissue repair, hepatic stellate cells (HSCs), a pericyte-like nonparenchymal liver cell population, transform from a quiescent status (resting HSCs) into myofibroblast like cells (activated HSCs); the latter is the principal matrix-synthesizing cell of the liver. Although several factors have been shown to be involved in this important process, the molecular mechanisms regulating HSC activation are still under investigation. To identify key regulatory proteins involved in the HSC activation process, we used different mRNA display technologies, with cDNAs prepared from HSCs at different stages of *in vitro* activation. With the latter technique, the transcription factor Ets-1 was detected through its down-regulation during activation. As confirmed by Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, mRNAs coding for Ets-1 were present in the highest amounts in freshly isolated HSCs and in HSCs 2 days after plating (classified as resting HSCs/early activated HSCs) and were diminished in HSCs 7 days after plating (activated cells). Ets-1 protein was present in HSC-lysates, as assessed by Western blot, and bound to an oligonucleotide containing the Ets-1 consensus *cis*-acting motif, as demonstrated by electrophoretic mobility shift assay. Ets-1 binding activity peaked in nuclear extracts prepared from resting/early activated cells and was diminished in extracts derived from fully activated cells. In contrast, binding activity of the transcription factors TFIID, AP-1, and SP-1 was highest in activated HSCs and only barely detectable in resting/early activated HSCs. By Northern blot and RT-PCR analysis, Ets-1-specific transcripts were present in parenchymal and other nonparenchymal liver cells too, illustrating that hepatic Ets-1 expression is not specific or

restricted to HSCs. However, the unique pattern of Ets-1 binding activity present in resting *versus* activated HSCs and its known implications for cellular differentiation and tissue remodeling suggest that Ets-1 could be of crucial importance for HSC activation and hepatic tissue repair. (Am J Pathol 1999, 155:1841–1848)

Hepatic stellate cells (HSCs) play a major role in vitamin A metabolism and are currently regarded as the principal cell type responsible for matrix accumulation during liver repair reactions, including fibrosis.^{1–3} A basic feature of the response of HSCs to hepatic tissue injury are phenotypic and functional changes, a process called activation.^{1–3} Activation includes HSC proliferation, transformation from star-shaped, vitamin A-rich cells to vitamin A-deficient cells with a myofibroblast-like appearance (activated HSCs) displaying contractile properties. Furthermore, activation is characterized by differential gene expression of connective tissue components, matrix-degrading enzymes, and their inhibitors, resulting in matrix accumulation colocalized with activated HSCs. Interestingly, this *in vivo* activation process strongly resembles the morphological and functional changes observed in HSCs during primary culture, and therefore HSCs *in vitro* are commonly used as a model to study the role of those cells during hepatic tissue repair.

Several extracellular stimuli, including, eg, inflammatory cytokines, growth factors, vasoactive peptides, and extracellular matrix components, as well as a number of intracellular signaling pathways, are involved in the activation process.^{4–6} However, the overall picture is far from complete, and the molecular mechanisms regulating HSC activation, particularly at the transcriptional level, are still under investigation. To address this question the present study used different mRNA display technologies

Supported by the Department of Medicine, University of Göttingen and by the Deutsche Forschungsgemeinschaft SFB 402 (Sonderforschungsbereich 402 "Molekulare und Zelluläre Hepatogastroenterologie").

Accepted for publication August 24, 1999.

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and cDNAs prepared from HSCs at different stages of *in vitro* activation to identify key regulators involved in this activation process. With the latter technique the transcription factor Ets-1 was detected through its down-regulation during HSC activation.

Ets-1 is the cellular homolog of the viral Ets oncogene of the E26 virus and functions as a sequence-specific transcription factor. It plays an important role in cell proliferation, differentiation, development, transformation, angiogenesis, and apoptosis.^{7,8} Ets-1 controls the expression of critical genes involved in these processes by binding to Ets binding sites present in their transcriptional regulatory regions. The Ets DNA-binding motif, GGA(A/T), has been found in numerous genes, including transcription factors, receptor-type kinases, and proteases. Among the proteases, stromelysins, collagenase, and urokinase plasminogen activator are typical Ets-1-responsive genes.^{9–11} Interestingly, all of the latter proteins are expressed by HSCs in the early phase of primary culture.^{12,13} Apart from direct DNA binding as monomers, Ets-1 cooperates with various transcriptional activators such as the AP-1 family in regulating gene activity⁷ and has been shown to activate gene transcription through a Ras-stimulated signal-transducing pathway that includes MAP kinases.^{14,15}

Because Ets-1 is of basic importance for cellular differentiation and because Ets-1-responsive genes were identified in hepatic stellate cells, the present study analyzed Ets-1 expression during the course of HSC activation. Ets-1-specific transcripts were studied by reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis, Ets-1 protein was analyzed by Western blot analysis, and Ets-1 binding activity was analyzed by electrophoretic mobility shift assay (EMSA) testing extracts prepared from HSCs at different stages of activation. In addition to Ets-1, nuclear extracts were analyzed for the presence of the general transcription factors AP-1, SP-1, and TFIID, demonstrating that Ets-1 DNA binding activity displayed a unique pattern during the course of HSC activation.

Materials and Methods

Animals

Wistar rats were provided by Charles River (Sulzfeld, Germany) and received humane care in compliance with the institution's guidelines and National Institutes of Health guidelines.

cDNA Probes

The Ets-1-specific c-DNAs generated by differential mRNA display technology or RT-PCR are described in the corresponding paragraphs. Furthermore, clone pFH154 coding for human fibronectin,¹⁶ a 2.7-kb rat glial fibrillary acidic protein (GFAP) cDNA clone,¹⁷ PCR-generated cDNAs directed against rat intercellular adhesion molecule-1 (I-CAM-1) and against rat vascular cell adhe-

sion molecule-1 (V-CAM-1),¹⁸ and a clone carrying the rat GAPDH cDNA were used.¹⁹

Antibodies

Rabbit polyclonal antibodies (C-20) raised against a peptide corresponding to amino acids 422–441 mapping at the carboxy terminus of Ets-1 of human origin and rabbit polyclonal antibodies (N-276) directed against amino acids 55–70 within the amino-terminal domain of human Ets-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies display no cross-reactivity with other Ets family members. The monoclonal antibodies against smooth muscle α -actin and β -actin as well as horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs were from Sigma (Munich, Germany).

Isolation and Cultivation of HSCs and Other Liver Cells

HSCs were isolated from rat liver and kept in primary culture as described previously.^{12,18,20–25} HSC at 2 days after plating displayed numerous vitamin A-containing vacuoles located around the nucleus and had a starlike appearance. At 7 days of primary culture HSCs showed a myofibroblast-like morphology characterized, eg, by cell enlargement and reduction of the size of intracellular vacuoles. By immunocytochemistry desmin expression was detectable in about 70% of the freshly isolated HSCs and in 100% of the cultivated HSCs. Although HSCs at 2 days after plating displayed a weak desmin-specific immunoreactivity, desmin-positive filaments became prominent in HSCs at later stages of cultivation, eg, at 7 days after plating. As assessed by morphology and by the expression of smooth muscle α -actin (SMA), glial fibrillary acidic protein (GFAP), and neural cell adhesion molecule (N-CAM), HSCs were fully "activated" at 7 days of primary culture, whereas cells cultured for 2–3 days were classified as "resting" HSCs/HSCs at an early stage of activation, and cells cultured for 4–5 days were judged as transitional cells.^{20,26}

Hepatocytes, Kupffer cells, and sinusoidal endothelial cells (ECs) were isolated from rat liver and cultivated as described previously.^{12,18,20–27}

Purity of freshly isolated cells and cultured cells was assessed as stated earlier.^{12,18,20–25} Relative purities of the freshly isolated cells were 98% (hepatocytes), 99% (Kupffer cells), 85% (HSCs; major contaminants were ECs and a few Kupffer cells), and 90% (ECs). HSC cultures showed only a <2% contamination with Kupffer cells; EC and hepatocytes were not detectable. Until day 3 of culture, in hepatocyte monolayers, less than 1% contaminating cells (mainly desmin and/or SMA-positive) were detected. EC cultures at day 3 after plating showed a 10% contamination with either desmin and/or SMA-positive cells or mononuclear cells. Kupffer cell primary cultures displayed less than 1% contaminating cells (mainly desmin and/or SMA-positive).

Rat liver myofibroblasts (rMFs) were obtained by outgrowth of primary nonparenchymal liver cells cultures. rMFs are characterized in detail elsewhere and have to be regarded as cells of the fibroblast lineage with similar but not identical phenotypic and functional properties compared to activated HSCs.²⁸ At passages 2 and 4 the rMF cultures showed no contamination with hepatocytes, Kupffer cells, ECs, or HSCs.

Western Blot Analysis

HSCs were lysed in hot Laemmli buffer (95°C)²⁹ and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using 7.5% or 9% resolving gels according to the method of Laemmli,²⁹ as described.^{20–22} The protein content of cellular lysates was calculated using the Coomassie protein assay (Pierce, Rockford, IL). Proteins were transferred onto Hybond-ECL-nitrocellulose hybridization transfer membranes according to the method of Towbin.³⁰ Immunodetection was performed according to the ECL Western blotting protocol of Amersham Buchler (Braunschweig, Germany).

RNA Extraction, RT-PCR, and Northern Blot Analysis

Cells were lysed with guanidinium isothiocyanate, and total RNA was extracted as reported.^{12,18,22,24,25}

For RT-PCR 1 µg of total RNA was reverse transcribed, using 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Karlsruhe, Germany) at 37°C. One microliter of a 20-µl total RT reaction was used as template DNA for PCR. PCR was performed with specific primers for 40 cycles with cycle times of 1 minute at 95°C, 1.30 minute at 55°C, and 2 minutes at 72°C. The final elongation time was 10 minutes at 72°C. The primer used to detect Ets-1 by RT-PCR had the following sequence: forward primer, 5' TAC CCT TCC GTC ATT CTC C 3', and reverse primer, 5' TTT TTC CTC TTT CCC CAT C 3', corresponding to positions 907–1433 of the rat sequence (data bank accession number L20681).³¹ Ten microliters of 50 µl total PCR reaction was analyzed in a 2% agarose gel. The specificity of PCR products was confirmed by sequencing of cloned PCR products. This PCR-generated cDNA probe gave the same Northern blot results as the original probe generated by random arbitrarily primed PCR (RAP-PCR). RT-PCRs for detection of GAPDH-specific transcripts were performed as described.²⁰

For Northern blot analysis 5 µg of total RNA was resolved by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with specific cDNA probes, which were ³²P-labeled. Hybridization was performed for 2 hours at 68°C, using the QuickHyb Kit (Stratagene, La Jolla, CA). Posthybridization washes were performed two times for 15 minutes at room temperature and one time for 5–15 minutes at 60°C in 2× standard saline

citrate containing 0.1% SDS. Nylon filters were washed, dried, and exposed to X-ray films at –80°C.

RAP-PCR, Cloning, and Sequencing

Differential m-RNA display technology was used as described,²⁴ using cDNAs prepared from HSCs at 2 and 7 days. Using the latter approach, Ets-1 was detected by RAP-PCR through its presence in samples derived from HSCs at day 2 after plating and its down-regulation from day 2 to day 7. The 635-bp-long cDNA fragment was generated by the use of a single 18-base arbitrary primer (primer sequence: 5' AAT CTA GAG CTC CAG CAG 3') and cloned into PCR2.1 cloning vector with the TA cloning kit (Invitrogen, San Diego, CA), and several clones were sequenced using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH). Sequence comparison was performed by FASTA, BestFit, or BlastN alignment programs, using standard parameters.³² The 635-bp-long fragment mapped to positions 674–1571 of the published rat Ets-1 cDNA (data bank accession number L20681).³¹

Preparation of Nuclear Extracts and Gel Mobility Assay

Nuclear extracts were prepared from HSCs directly after isolation⁰ and at days 2¹ and 7³ after plating as described.³³ Briefly, cells were incubated in extraction buffer (20 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride) for 15 minutes on ice, followed by the addition of 10% Nonidet NP-40. After centrifugation the pellet was resuspended in extraction buffer containing 400 mmol/L NaCl, agitated vigorously for 15 minutes at 4°C, and centrifuged for 5 minutes. The supernatant was recovered, and the protein content of the nuclear extracts was calculated using the Coomassie protein assay (Pierce).

Nuclear extracts were incubated with ³²P-labeled double-stranded oligonucleotides encoding the wild-type Ets-1 binding sequence (5' GTC AGT TAA GCA GGA AGT GAC TAA C 3'; underlined bases are required for Ets-1 binding) or a mutant binding sequence (5' GTC AGT TAA GCA GGC AGT GAC TAA C 3', underlined base is exchanged) according to the method of Hultgardh-Nilsson et al.³⁴ In addition, oligonucleotides encoding the binding sequences for AP-1 (5' CGC TTG ATG AGT CAG CCG GAA 3'), SP-1 (5' ATT CGA TCG GGG CGG GGC GAG C 3'), and TFIID (5' GCA GAG CAT ATA AGG TGA GGT AGG 3') were tested. Binding of the nuclear extracts to the oligonucleotide probe (8000 cpm) was performed in binding buffer (20 mmol/L HEPES, 25 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.25 mmol/L phenylmethylsulfonyl fluoride) at room temperature for 30 minutes as described.³⁵ DNA-protein complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis in 0.5× TBE and detected by autoradiography.

Figure 1. Ets-1 expression during *in vitro* activation of hepatic stellate cells. **A:** Expression of Ets-1 as assessed by Northern blot analysis. Total RNA was purified from freshly isolated HSC (0), HSC at days 2 or 3 (2/3), 4 or 5 (4/5), or 7 (7) after plating. For Northern blot analysis RNA prepared from four different cell preparations was pooled to overcome individual differences. Total RNA (1.25 μ g) recovered from four different HSC isolations was pooled and size selected by 1% agarose gel electrophoresis, followed by hybridization using specific cDNA probes. **B:** Detection of Ets-1-specific messengers by RT-PCR. Total RNA isolated from HSC directly after isolation (0) and at days 2 (2) and 7 (7) after plating was reverse transcribed and analyzed by amplification with PCR, using Ets-1 specific primers. M: bp weight markers (123 bp ladder). **C:** Presence of Ets-1 protein in HSC-derived cellular lysates. HSCs at day 2 (1) or 7 (2) after plating were solubilized in hot Laemmli buffer, and 10 μ g of cellular proteins was analyzed by 7.5% SDS-PAGE followed by Western blotting, using antibodies directed against Ets-1, smooth muscle α actin (SM α -actin), or β -actin.

Results

Expression of Ets-1 by Hepatic Stellate Cells in Vitro

Employing differential mRNA display technology, we detected a 635-bp-long cDNA fragment through its presence in samples derived from HSCs at day 2 after plating and its down-regulation from day 2 to day 7. This fragment mapped to the rat Ets-1 cDNA positions 674–1571 (data bank accession number L20681), in more detail to a spliced variant lacking exon VII located at 1024–1286.³¹ Sequence comparison of the PCR product with the rat Ets-1 c-DNA (data bank accession number L20681) illustrated 98% homology. Mismatches mapped to the 18-base arbitrary primer, especially the one located at the 3' end, and were additionally distributed randomly within the sequence, which is probably due to misreading of the enzyme used for PCR and sequencing. With this cDNA fragment as a probe for Northern blot hybridization, Ets-1-specific messengers of 5.3 kb and 2.3 kb in size, known to arise from differential polyadenylation,³¹ were shown to be present in HSCs in accordance with the literature^{31,34,36} (Figure 1A). In addition, a third Ets-1-specific transcript 4.0 kb in size³⁴ was present, which is barely visible in Figure 1A but is detectable after prolonged exposure.

To analyze the rate of the differential splicing of exon VII, primers were constructed, which were located upstream and downstream of exon VII, thereby giving rise to PCR products 527 bp in size in the case of the presence of exon VIII and to 265-bp-long PCR products in the case of the absence of exon VII. As demonstrated in Figure 1B, predominantly 527-bp-long products were

present in the RT-PCR analysis, thereby indicating that the majority of the Ets-1 coding messengers present in HSCs contained exon VII and that alternative splicing of exon VII is a rare event, as observed in other systems.³⁷ Using this RT-PCR-generated Ets-1-specific probe for Northern blot analysis, we noted the same hybridization pattern as in the case with the probe cloned by differential mRNA display (data not shown).

Using specific antibodies, we detected Ets-1 protein in HSC-derived cellular lysates at about 54 kD, as assessed by SDS-PAGE and Western blot analysis (Figure 1C). Both the antibody C-20 directed against the carboxy terminus of Ets-1 and the antibody N-276 directed against the amino-terminal domain of Ets-1 were reactive; however, reactivity of the antibody C-20 was stronger, and therefore the latter antibody was used. In addition to the 54-kD Ets-1 band, a second band of slightly higher molecular weight was present (Figure 1C, asterisk), which was also observed by others using the C-20 antibody in the rat system³⁸ and might correspond to Ets-1 isoforms arising by different phosphorylation.³⁹

The DNA binding activity of Ets-1 protein present in HSCs was analyzed by EMSA illustrating specific DNA–Ets-1 complexes (Figure 2A). The specificity of these complexes was provided by the use of an oligonucleotide carrying a mutant Ets-1 binding site, which resulted in the disappearance of DNA–Ets-1 complexes (Figure 2A, lane 1). Furthermore, specificity was proved by adding increasing amounts of nuclear extracts, which resulted in an increase in shifted complexes (Figure 2A, lanes 2 and 3), or by a reduction of the DNA–Ets-1 complexes through the addition of unlabeled oligo (Figure 2A, lane 5).

Figure 2. Activity of Ets-1 protein in resting and activated HSCs as assessed by electrophoretic mobility shift assay (EMSA). **A:** Ets-1-specific binding activity of nuclear extracts. Nuclear extracts were prepared from HSCs at day 2 after plating and were incubated with either mutated Ets-1 oligonucleotide (1) or with the wild-type Ets-1 binding motif (2–5). In lane 2, 1 μ g of nuclear extract was incubated, and in lanes 1, 3, 4, and 5, 2 μ g of nuclear extracts was used. Incubation was performed in the presence (5) or absence (4) of 116 μ mol/L unlabeled wild-type Ets-1 binding motif. **B–D:** DNA binding activity of extracts from HSCs at different stages of *in vitro* activation. Nuclear extracts were prepared from HSCs directly after isolation (0) and at days 2 (2) and 7 (7) after plating. Two-microgram extracts were incubated with oligonucleotides carrying the binding sites of Ets-1 (**B**), TFIID (**C**), AP-1 (**D**), or SP-1 (**E**).

Ets-1 Expression and Ets-1 DNA Binding Activity during Activation of Hepatic Stellate Cells

Comparing cells of day 2 with cells of day 7 after plating, we detected Ets-1 expression of HSCs initially by mRNA differential display through its down-regulation. As confirmed by Northern blot (Figure 1A) and RT-PCR analysis (Figure 1B), mRNAs coding for Ets-1 as well as Ets-1 protein present in cellular lysates (Figure 1C) were present in higher amounts in HSCs 2 days after plating (classified as resting HSCs/early activated HSCs) compared to HSCs 7 days after plating (classified as fully activated cells). Incorporating freshly isolated cells in the analysis demonstrated that even higher amounts of Ets-1 coding messengers were present in HSCs directly after isolation (regarded as resting cells) (Figure 1, A and B). Control hybridizations using well-established marker proteins^{20,26,40} demonstrated that in the same set of experiments GFAP expression is diminished during the course of HSC activation (Figure 1A), that mRNAs coding for the extracellular matrix protein fibronectin are elevated in activated HSCs compared to resting cells or transitional cells (HSCs at day 4–5 after plating) (Figure 1A), and that

the classical activation marker smooth muscle α -actin is present in major amounts in fully activated cells (Figure 1C).

As demonstrated by EMSA (Figure 2B), Ets-1 binding activity peaked in nuclear extracts prepared from resting/early activated cells (Figure 2B, lanes 0 and 2) and was diminished in extracts derived from fully activated cells (Figure 2B, lane 7). In contrast, when we used identical nuclear extracts, binding activity of the transcription factors TFIID, AP-1, and SP-1 was highest in activated HSCs and only barely detectable in resting/early activated HSCs (Figure 2, C–E).

Ets-1 Expression among Different Liver Cell Populations in Vitro

To examine whether Ets-1 expression in the liver is restricted to HSCs, other liver cell populations were studied by Northern blot and RT-PCR analysis (Figure 3). By RT-PCR analysis Ets-1-specific messengers were detected in all liver cell types (Figure 3A). As in the case of HSCs, mRNAs containing exon VII were the predominant form in other liver cell types. Hybridization of filters containing RNA samples derived from hepatocytes, Kupffer cells, ECs, and rMFs confirmed the latter results and demonstrated that based on a microgram RNA basis, Ets-1 expression was prominent in HSCs and ECs but was also present in considerable amounts in hepatocytes (Figure 3B). Control hybridizations were performed against I-CAM-1, which is known to be present in all liver cell types, and V-CAM-1, which is expressed in major quantities in HSCs.^{18,27} Apart from the fact that Figure 3B definitely indicates that Ets-1 expression of HSCs is not derived from contaminating cells, the Northern blot results pointed out that Ets-1 expression is not restricted to HSCs. In addition, the data demonstrate that also in the case of ECs a dramatic down-regulation of Ets-1 expression occurred on cultivation, resembling the pattern described for HSCs, and that in the case of hepatocytes Ets-1-specific signals were present in the highest quantities after cultivation.

Discussion

The data presented in this report illustrate that HSCs express the transcription factor Ets-1 as evidenced by RT-PCR, by Northern and Western blot analysis, as well as by EMSA. Ets-1 expression by HSCs was studied at various time points of primary culture reflecting different stages of HSC activation. In the latter approach, the data demonstrate that HSCs in both the resting and activated states express Ets-1; however, levels of Ets-1-specific transcripts, of Ets-1 protein as well as Ets-1-specific DNA binding activity, were highest in freshly isolated and early cultured HSCs (classified as resting/early activated cells) and were diminished severalfold during the course of transformation to fully activated cells.

The Ets-1 expression pattern of HSCs is quite unique compared to the transcription factors described so far in

Figure 3. Expression of Ets-1 among different types of liver cells. **A:** RT-PCR. Total RNA was isolated from Kupffer cells (KC) directly after isolation (0) and at days 1 (1) and 3 (3) after plating; from hepatocytes (HEP) directly after isolation (0) and at day 1 (1) and day 3 of culture (3); from sinusoidal endothelial cells (EC) directly after isolation (0) and at day 3 (3) after plating; from rat liver myofibroblasts (rMF) of passages 2 (2), 4 (4), and 6 (6); and from HSCs directly after isolation (0) and at days 2 (2) and 7 (7) after plating. Hepatocytes analyzed by RT-PCR were cultured without dexamethasone addition. **B:** Northern blot analysis. Total RNA was purified from rat liver myofibroblasts (rMF) of passages 2 (2) and 4 (4); from HSCs directly after isolation (0) and at days 2 (2) and 7 (7) after plating; from KCs directly after isolation (0) and at days 1 (1) or 3 (3) of primary culture; from sinusoidal endothelial cells (EC) directly after isolation (0) and at day 3 (3) after plating; and from hepatocytes directly after isolation (0) and at days 1 (1) and 3 (3) after plating. Hepatocytes were cultured with (+) or without (−) 10^{-7} mol/L dexamethasone. Five micrograms of total RNA was size selected by 1% agarose gel electrophoresis, and filters were hybridized using specific cDNA probes.

HSCs like AP-1,^{41–44} SP-1,⁴⁵ CCAAT binding factor (CBF),⁴⁵ NF-kappaBs⁴⁶ and the recently identified novel, kruppel-like transcription factor Zf9.⁴⁷ Whereas the latter transcription factors as well as TFIID presented in this study were induced during HSC activation, Ets-1 expression was diminished in fully activated cells, thereby resembling the pattern of the novel 75-kd transcription factor (P75), which was recently identified in HSCs and which might act as an E-box-binding repressor.⁴⁸

One might speculate that the isolation process caused an induction of Ets-1 expression, thereby suggesting that Ets-1 expression of HSCs directly after preparation is unrelated to the differentiation stage. Although the authors cannot exclude this possibility, the lack of Ets-1 induction (eg, in freshly isolated hepatocytes) argues against this concept. However, the results obtained with cultured cells clearly indicate that Ets-1 expression is dependent on the activation state of HSC. The high Ets-1

expression level of cells cultured for 2 days and its down-regulation during the transformation to fully activated cells later on indicate that Ets-1 expression is present at a very early stage of activation but is diminished on further transformation. Interestingly, the high Ets-1 expression levels present in early cultured HSCs (2 days after plating) coincide with a transient up-regulation of typical Ets-1-responsive genes like stromelysins, collagenase, and urokinase plasminogen activator in HSCs at this time point of primary culture,^{12,13} thereby demonstrating that Ets-1 expression is indeed accompanied by the activation of the corresponding genes.

To test whether the down-regulation of Ets-1 during the transformation of HSCs to fully activated cells also occurs *in vivo*, activated HSCs were isolated from carbon tetrachloride-injured livers to compare them with resting HSCs isolated from normal livers. In accordance with our previous unpublished findings, we were unable to obtain HSCs from injured livers at a purity comparable to that of cells isolated from normal liver. As other liver cells also express Ets-1, highly purified HSC preparations are necessary to analyze Ets-1 expression in a cell-type-specific manner, and therefore the demonstration of Ets-1 down-regulation in activated HSCs *versus* resting HSCs was not possible with this approach.

Furthermore, we also performed dual immunohistochemistry of normal *versus* injured livers, using antibodies directed against Ets-1 and desmin. Because of the sensitivity of the antibody and the fact that all other liver cells were Ets-1-positive at some stage, the data obtained by this technique were also not useful for demonstrating that Ets-1 expression is down-regulated during HSC activation *in vivo*. In summary, as both latter approaches were not useful for analysis of Ets-1 expression of HSC *in vivo*, *in situ* hybridization combined with immunohistochemistry is necessary to determine whether Ets-1 down-regulation occurs during the transformation of HSCs to fully activated cells *in vivo*.

Furthermore, the data presented in this study illustrate that hepatic Ets-1 expression is not restricted to HSCs and indicate for the first time that Ets-1 is also detectable in other liver populations, especially in sinusoidal endothelial cells and hepatocytes apart from hepatoma cell lines reported previously.^{49,50} Ets-1 expression by HSCs and endothelial cells are in line with the observation that in the adult, Ets-1 is expressed predominantly in lymphoid cells⁵¹ but is also present in mesoderm lineage cells, including endothelial cells and fibroblasts.^{52,53}

The question arises about the physiological role of Ets-1 expression by HSCs. Ets proteins are important components of the transcription machinery and thereby regulate the expression of several genes encoding extracellular matrix proteins as well as enzymes involved in matrix degradation.⁷ Ets activity is affected by a variety of growth modulators and mitogenic signals, and a role of Ets-1 in activation of DNA replication has been documented. During tissue injury Ets-1 activity is transiently induced during the early stages of tissue remodeling (eg, in the early phase of ulcer healing)³⁸ or immediately after mechanical injury of the vessel wall. Ets-1 expression was detected in the latter models in endothelial cells, smooth

muscle cells, and fibroblasts,^{34,54} suggesting a role for Ets-1 in cell migration and cell replication.³⁷ Based on these data, it is tempting to speculate that Ets-1 plays a similar role in HSCs at an early stage of cell activation (2 days after plating). Ets-1 expression might be critical to the events mapping to this early activation stage in detail, to the onset of cell proliferation, and to enhanced expression of matrix-degrading enzymes, thereby suggesting a key role for Ets-1 in the initiation of HSC activation.

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