Differences in regulation of type I collagen synthesis in primary and passaged hepatic stellate cell cultures: the role of α5β1-integrin

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Differences in regulation of type I collagen synthesis in primary and passaged hepatic stellate cell cultures; the role of α5β1-integrin. Am J Physiol Gastrointest Liver Physiol 293: G154–G164, 2007. First published May 17, 2007; doi:10.1152/ajpgi.00432.2006.—Hepatic stellate cells (HSC) differ in their phenotype depending on the initiation and progression of their activation. Our hypothesis was that different mechanisms govern type I collagen synthesis depending on stage of HSC activation. We investigated the role of α5β1-integrin as a regulator of type I collagen gene COL1A1 expression in primary and passaged HSC cultures using transgenic mouse containing type I collagen gene COL1A1 promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene. The α5β1 protein levels increased during the activation and were highest in day 6 primary cultures but decreased in passaged HSC. CAT activity, reflecting COL1A1 expression, was upregulated by α5β1-integrin. Inhibition of α5β1-integrin by echistatin and blocking antibody resulted in reduced transgene activity only in early primary cultures (compared with the control, 53.3 ± 12% echistatin and 58.8 ± 7% blocking antibody, respectively, P < 0.05). Treatment of passaged HSC with either echistatin or blocking antibody had no effect. Fibronectin, an α5β1-integrin ligand, increased transgene activity in primary (210 ± 33%, P < 0.05) but not in passaged HSC cultures (119 ± 8%). This α5β1-integrin effect appears to be at least in part mediated by CCAAT enhancer binding protein-β (C/EBPβ), because fibronectin increased and α5-gene silencing by small interfering RNA decreased C/EBPβ levels. In addition, C/EBPβ knockout mice showed reduced type I collagen synthesis compared with wild-type littermates. Therefore α5β1-integrin is an important regulator of type I collagen production in early primary HSC cultures but appears to have no direct role once the HSC are fully activated.

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through the fibronectin receptor, α5β1-integrin, which plays a role in both HSC attachment to fibronectin and in the activation process (16, 30). Additional evidence for this role of α5β1-integrin in HSC activation is derived from studies on blockade of α5β1-integrin by Arg-Gly-Asp peptides that ameliorates CCL4-induced liver fibrosis (21).

Integrins are adhesion receptors located on the cell surface and the major membrane proteins involved in cell-ECM interactions (8). Each integrin is a heterodimer consisting of non-covalently bound α- and β-subunits. The ligand specificity depends primarily on the extracellular component of the α-subunit. However, both subunits are required for an integrin to function. Multiple integrins have been reported to exist on the HSC membrane, including the fibronectin receptor α5β1-integrin (4). Integrins have also been implicated as regulators of type I collagen synthesis in other tissues and organs (1, 9).

The mechanism of increased expression of type I collagen COL1A1 gene by α5β1-integrin is unclear. We hypothesized that α5β1-mediated signaling may upregulate expression of a transcriptional enhancer in activating HSC. One such transcription factor previously described to play a role in a type I collagen expression is CCAAT enhancer binding protein-β (C/EBPβ). In HSC, C/EBPβ has been reported to mediate acetaldehyde and hydrogen peroxide-induced upregulation of type I collagen transcription (2, 11).

In the present study we examined the relationship between α5β1-integrin and type I collagen expression in primary (day 6) and passaged HSC cultures. Our data suggest that type I collagen expression is regulated by different mechanisms, depending on the phase of HSC activation. α5β1-Integrin is important for high levels of type I collagen expression in primary cultures. However, passaged HSC do not require either fibronectin or α5β1-integrin signaling to maintain high levels of type I collagen expression. Our data also suggest that the α5β1-integrin effect on type I collagen synthesis in primary HSC cultures is in part mediated by the transcription factor C/EBPβ.

MATERIALS AND METHODS

Animals. Transgenic mice harboring construct pOBColCAT3.6 were used to evaluate type I collagen expression in HSC. pOBColCAT3.6 contains 3.6 kb of rat type I collagen gene COL1A1 promoter with parts of the first intron, driving expression of the reporter gene chloramphenicol acetyltransferase (CAT). Another transgenic line harboring a similar construct containing the same upstream regulatory COL1A1 promoter sequence but with green fluorescence protein as a reporter gene (pOBColGFp3.6) was used to evaluate number of type I collagen-expressing cells in the culture upon activation. Transgenic mice and measurements of reporter gene were used to focus on changes in COL1A1 gene expression independent of any posttranscriptional and posttranslational processing involved in deposition of type I collagen by HSC. The use of transgenic animals simplified type I collagen expression measurement and permits quantification of changes following experimental manipulations. Animals were kindly provided by Dr. David Rowe and Dr. Alexander Lichtler from University of Connecticut Health Center, Farmington, CT. These transgenic lines have been characterized previously, and promoter constructs have been shown to support endogenous type I collagen like tissue expression pattern (3, 17). In addition, our data indicate that changes in the reporter gene closely resemble changes in mature collagen synthesis estimated by Western blot analysis of the growth media.

C/EBPβ −/− mice were used to investigate the role of C/EBPβ in HSC activation and resultant type I collagen transcription. C/EBPβ −/− mice and heterozygous (wild-type phenotype) littermates were generated by Dr. Valeria Poli (6) and provided by Dr. Colleen M. Croniger and Dr. Richard W. Hanson from Case Western Reserve University, Cleveland, OH. The generation of C/EBPβ −/− mice and their genetic background has been described previously (6).

All animal experiments were reviewed and approved by Institutional Animal Care and Use Committee at Case Western Reserve University, Cleveland, OH.

HSC isolation and culture. Primary HSC were isolated from the liver as previously described (13). Briefly, after animals were killed, livers were removed, washed in ice-cold HBSS (HyClone, Logan, UT), minced, and incubated in HBSS without calcium and magnesium with 0.2% Pronase (Sigma, St. Louis, MO) and 0.02% Collagenase H (Sigma) for 30 min at 37°C. The digest was passed through a fine wire mesh and the resultant cell suspension was washed three times with HBSS. HSC were further purified by density gradient centrifugation. Cells were resuspended in HBSS with 3% BSA (Sigma) and mixed with Histodenz (Sigma) in HBSS without NaCl. This mixture was then layered under HBSS with 3% BSA. After centrifugation, the white interphase layer containing HSC was removed, washed, and plated at 250,000 cells/cm² in DMEM with 10% fetal calf serum and 10% horse serum. All media and sera for cell culture were purchased from HyClone (Logan, UT). The medium was changed daily during the experiment. Cells were passaged on days 7-8 of primary cultures and plated at 25,000/cm² density. These cells were actively replicating without signs of replicative senescence, secreted large amount of type I collagen, and exhibited all other features of the fully activated phenotype.

The purity of the cultures was evaluated by morphology, vitamin A autofluorescence at 328 nm on day 1 of culture, α-smooth muscle actin (αSMA), desmin, and glial fibrillary acidic protein (GFAP) immunofluorescence. In addition, activated cells isolated from 3.6ColGFP transgenic mice were examined for fluorescence daily during the culture period.

Fibronectin (Sigma) was used to coat culture dishes in a concentration of 10 μg/ml for 24 h before subcultures of either primary or secondary HSC cultures were plated. Echistatin (Sigma) in doses of 100, 10, and 1 nM, as well as αβ1 blocking antibody (Chemicon, Temecula, CA) (10 μg/ml), was added to primary cultures on day 4, and cells were harvested after 24 h. For passaged HSC, echistatin and α5β1 blocking antibody were added to secondary cultures 3 days after passage.

α5-integrin gene silencing in HSC culture. HSC were isolated as described above. At 24 h after plating, HSC were transfected with 10 nM of α5-integrin small interfering RNA (siRNA) in HiPerFect transfection reagent. Cells were harvested and analyzed at day 5 of culture. The α5-integrin, type I collagen, and C/EBPβ protein levels were determined in the cell lysate. Both α5-integrin siRNA and transfection reagent were purchased from Qiagen (Valencia, CA) and used according to the manufacturer’s recommendations.

RNA isolation and real-time RT-PCR. Total RNA was isolated using TRI reagent (Sigma). Reverse transcriptase kit was purchased from Invitrogen (Invitrogen, Carlsbad, CA) and used to generate cDNA per manufacturer recommendations. Specific primers were designed and analyzed by use of commercial software (Light Cycler Probe Design, Roche Diagnostics). The primer sequences for α5-integrin are as follows: α5-forward primer 5′-ACCAAGACGGCC-TACAATGTAG-3′ and α5-reverse primer 5′-CTGCTTTGGAAGT-CAGGACAC-3′. The GAPDH primers are as follows: GAPDH forward 5′-GTGAGCATCAAGAGGTTGGTA-3′ and GAPDH reverse 5′-GGTCCAGGGTTTTCTACTCCT-3′. Primers for COL1A1
gene were COL1A1 forward 5′-AGTTCAGCTTTGTTGACCTC-3′ and COL1A1 reverse 5′-AGTTGAGACGACACTCGG-3′. Real-time PCR for quantification of RNA was carried out by using SYBR protocol on the fluorescence temperature cycler (Light Cycler; Roche Molecular Diagnostics, Indianapolis, IN). The reaction conditions were optimized at different temperature ranges and magnesium concentrations. Real-time reactions were carried out in duplicate, and amplicons were analyzed by generating melting curves with continuous measurement of fluorescence. Results were calculated as relative differences in target threshold cycle values normalized to GAPDH. All real-time PCR products were separated on a 1.5% Tris-acetate agarose gel to confirm product presence and size. Values were expressed as fold increase in the target mRNA at days 4, 6, and 8 of primary culture compared with day 2.

CAT assay. CAT assay was performed by using a standard fluor diffusion protocol. Cells were scaped from the culture in 0.3 ml of extraction buffer (0.25 M Tris-HCl, pH 7.8, containing 0.5% Triton X-100), subjected to three cycles of freezing and thawing, and then heated at 65°C for 15 min to inactivate endogenous deactylases. After centrifugation, the supernatants were used to determine CAT activity and protein concentration. CAT activity was measured by a modified fluor diffusion assay (23) and normalized to the protein content of the extract as measured by the BCA assay (Pierce, Rockford, IL).

Proliferation assay. Proliferation of the HSC was measured with Alamar blue (Serotec, Westbury, NY) according to manufacturer recommendations. We added 900 μl of fresh medium and 100 μl of Alamar blue to 12-well cell culture plates, mixed gently, and incubated for 4 h. Cell proliferation was determined by a colorimetric change in the medium measured as a difference in culture media absorbance at 570 and 600 nm. Using primary and passaged HSC cultures, we demonstrated that this method showed a high correlation with the total cell number in culture (r² = 0.992, P < 0.01) and bromodeoxyuridine incorporation (r² = 0.998, P < 0.001) (data not shown). This corresponded to the manufacturer’s data supplied in the Alamar Blue Datasheet and previously published studies (7).

Protein isolation and Western blot analysis. Primary HSC cultures on days 2, 3, 4, 5, 6, 7, and 9, as well as passaged HSC cultures, were lysed with ice cold lysis buffer containing 25 mM Tris pH 7.4, 50 mM NaCl, 25 mM NaF, 10% glycerol, 1% Triton X-100, PMSF (1 mM), protease inhibitors cocktails 10 μM/ml (Sigma no. P8340, Sigma); scraped; and left on ice for 30 min. Protein concentration was determined by DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein samples were resolved on a 7.5% SDS-PAGE under reducing conditions and then electrotransferred to polyvinylidene difluoride membrane. For α5β1-integrin analysis, the membrane was blocked with 5% nonfat milk and 2% BSA in TBS with 0.1% Tween, incubated with goat polyclonal antibody to α5β1 (Chemicon, Temecula, CA), washed, and then incubated with horseradish peroxidase-conjugated bovine anti-goat IgG antibody (Santa Cruz Biotechnology); the membrane was subsequently incubated with rabbit polyclonal antibody to α5β1-integrin, washed, and then incubated with horseradish peroxidase-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology). The blots were visualized with the ECL detection (Amersham, Piscataway, NJ). A similar procedure was followed for Western blot analysis for C/EBPβ protein (antibody SC-150X, Santa Cruz Biotechnology); the only difference was that samples were loaded on 12% SDS polyacrylamide gel.

Western blot for type I collagen was performed on cellular extracts as well as on harvested growth media. Cellular extracts from HSC were prepared by lysing HSC at designated time points in 2× sample buffer [0.125 M Tris pH 7.0, 4% SDS, 20% glycerol, 1 mM PMSF, 10 μM protease inhibitor cocktail (Sigma), 2 mM activated Naorthovanadate]. For collection of growth medium, cells were incubated in serum-free DMEM enriched with 50 μg/ml ascorbic acid (Sigma) for 24 h. The medium was collected and spun through Ultracel YM-10 centrifugal filter unit (Millipore, Billerica, MA). After measurement of protein concentration, 1 ng of total protein was precipitated and loaded on 7.5% SDS polyacrylamide gel. Further Western blot analysis was performed as outlined above. Antibody against mature type I collagen (SC-8788) was used for analysis of growth media, and antibody against type I procollagen (SC-8787) was used for analysis of cell extracts.

Immunostaining. Desmin, αSMA, and GFAP antibodies for immunofluorescence were purchased from Sigma. HSC were grown on polylysine-treated slides and fixed in 3.7% formaldehyde in PBS. Slides were incubated in primary antibody overnight at 4°C. After incubation with secondary antibody, labeled with fluorophore, immunofluorescence was visualized by fluorescent microscope. For negative control we used serum corresponding to the primary antibody source.

RESULTS

Characterization of HSC cultures. HSC isolated from transgenic mice were identified according to their morphology and ability to synthesize αSMA. The purity of primary cultures, assessed by morphology and vitamin A autofluorescence measured in six independent cultures, was 85.2 ± 1.2% of the total cells in culture. Vitamin A autofluorescence and at phase photomicrographs are shown in Fig. 1, A and B. As seen in Fig. 1C, primary HSC on day 6 of culture were positive for GFAP, in accordance with their stellate cell phenotype. Day 8 primary cultures were strongly positive for αSMA, a marker of activated stellate cell phenotype (Fig. 1D). αSMA immunofluorescence also suggests that by day 8 of culture HSC represent more than 90% of cells in culture. In addition to these markers, we also stained passaged cells for desmin, another marker of HSC, reported to be positive in rodent HSC regardless of activation status. As seen in Fig. 1, E and F, most of the cells in passaged cultures were positive for desmin. GFAP (for early stages of activation) and desmin (independent of HSC activation status) were previously described as markers of HSC lineage, almost completely absent from liver fibroblasts (20).

HSC isolated from transgenic line harboring 3.6ColGFP demonstrated fluorescence once they became activated and synthesis of type I collagen was initiated. There was a gradual development of the fluorescence in cells, as well as a dramatic increase in number of fluorescent cells after 6–7 days in the culture, indicative of progressive activation of HSC and consequent type I collagen expression by cultured cells (Fig. 2).

The time course of CAT transgene, representing COL1A1 promoter activity (HSC isolated from transgenic line harboring pOB3.6COLCAT construct where CAT reporter gene is driven by the same COL1A1 promoter as in pOB3.6COLGFP transgenic mice), showed minimal activity in the first 3 days of culture. CAT levels in cultures harvested on day 3 were not different from the background. COL1A1 promoter activity was consistently higher on day 5 of culture and increased further as activation progressed. Maximal activity was observed at day 7.
As shown in Fig. 4A, day 2 HSC cultures from unit. C: immunofluorescence staining for glial fibrillary acidic protein (GFAP), an early HSC marker, along with nuclear staining, at day 6 of primary culture (magnification ×100). D: immunofluorescence for α-smooth muscle actin (αSMA; red), marker of activated HSC phenotype, at day 10 of culture (magnification ×100). Majority of cells showed presence of αSMA filaments in cytoplasm, suggestive of activated HSC phenotype. E: same slide as seen in D, stained for GFAP (green), along with nuclear staining, shows majority of αSMA-positive cells also stain positive for GFAP, implicating stellate cell origin of myofibroblasts. F: composite double image of D and E, showing composite color (yellow-orange) in majority of visualized cells, implicating that visualized cells are positive for both αSMA and GFAP. G and H: immunofluorescence for desmin, HSC marker independent of activation status, in passaged cultures of HSC. G: low-power (×100) photomicrograph showing majority of cells in passaged cultures are positive for desmin. H: higher power (×200) photomicrograph showing typical filamentous cytoskeletal features of desmin.

and plateaued thereafter (Fig. 3A). This increase of COL1A1 promoter activity during activation mimics the endogenous COL1A1 gene expression that we observed in the primary cultures using real time quantitative RT-PCR (Fig. 3B). It also parallels levels of endogenous type I collagen protein, as seen on the Western blot of the growth media from quiescent (day 3) and activated (day 7) HSC (Fig. 3C).

**HSC activation and α5β1-integrin.** Levels of α5β1-integrin have been reported to increase during the HSC activation, but its kinetics in the later stages of activation is largely unknown. As shown in Fig. 4A, Western blot analysis revealed that α5β1-integrin levels increase as HSC activate; however, once activated (days 6 and 7), the amount of α5β1-integrin decreases to the levels found in quiescent HSC. Low levels are observed in passaged cultures of HSC, as well as in “older” primary cultures (day 9), indicating that the decrease in the α5β1-integrin is not an artifact related to passage. There was an increase in the expression of α5-integrin mRNA in primary HSC cultures from day 2 to day 6, with decrease on day 8 (Fig. 4B), that paralleled the changes in α5-integrin protein levels. We chose to investigate α5-mRNA rather than β1-integrin because α5-dimerizes only with β1-integrin subunit. β1-integrin, on the other hand, dimerizes with multiple other integrin subunits and changes in its expression could reflect events different than those mediating α5β1-integrin changes. Furthermore, β1-integrin protein levels remained constant through the culture period, showing no difference between quiescent HSC, early primary cultures, and passaged HSC (data not shown). Changes in the levels of α5-integrin protein, however, closely paralleled the pattern observed with α5β1-integrin (Fig. 4C).

To investigate the role of α5β1-integrin in HSC activation and type I collagen expression, we treated primary HSC (days 4-6 of primary culture) with echistatin, an α5β1-integrin blocker (45). Primary HSC cultures treated with echistatin showed a significant dose-dependent decrease in COL1A1 promoter activity (P < 0.05; Fig. 5A). A similar decrease was observed in the amount of secreted endogenous type I collagen protein (Fig. 5B). Decreased COL1A1 promoter activity was also observed in the primary HSC (day 6 culture) with the use of α5β1-integrin blocking antibodies. Echistatin and α5β1-integrin blocking antibody did not significantly affect COL1A1 promoter activity in secondary (passaged) HSC cultures (Fig. 5C). Secondary cultures were treated 3 days after passage to allow cells to recover most of their membrane components that may have been damaged during trypsin treatment of the primary cultures. Proliferation was not significantly affected by echistatin treatment in either primary or passaged HSC cultures (data not shown).

The next series of experiments was designed to investigate the effect of fibronectin, the natural α5β1-integrin ligand, on proliferation and COL1A1 promoter activity of the primary (day 6) and passaged HSC. Both primary and passaged HSC were plated in wells previously coated with fibronectin. Fibronectin stimulated COL1A1 promoter activity in primary HSC cultures (day 6) (Fig. 6A). In contrast, COL1A1 promoter...
activity of the passaged HSC cultures was not affected by fibronectin (Fig. 6B).

These data suggest that type I collagen synthesis is regulated differently in early primary HSC cultures compared with the HSC in the fully established activated state represented by passaged cultures. HSC early in their activation utilize signaling through α5β1-integrin to increase their type I collagen synthesis. That pathway is likely activated by increased fibronectin synthesis and deposition in the ECM surrounding HSC early in liver injury. Once HSC are fully activated, fibronectin and α5β1-integrin do not appear to play a significant role in regulation of type I collagen synthesis.

C/EBPβ and type I collagen synthesis. C/EBPβ is a transcription factor reported to be involved in the control of type I collagen gene expression in HSC. In addition, C/EBPβ has been implicated in regulation of α5-integrin gene expression. This regulation was cell type specific, as C/EBPβ was repressor of α5-promoter activity in keratinocytes but stimulator in hepatoma HepG2 cells (5). C/EBPβ mRNA is translated into two principal isoforms of C/EBPβ: liver-enriched activating protein (LAP, ~35 kDa) and liver-enriched inhibitory protein (LIP, ~20 kDa). As shown in Fig. 7A, there was an increase in C/EBPβ in primary HSC culture on days 5 and 7. C/EBPβ paralleled changes in α5β1-integrin described earlier. On day 2 of primary culture (mostly quiescent phenotype) as well as in passaged HSC cultures, C/EBPβ levels were lower than in primary HSC cultures (days 5 and 7) (Fig. 7A). HSC plated on fibronectin showed increased levels of C/EBPβ protein on day 6 of primary culture, further supporting a possible role of C/EBPβ in fibronectin/α5β1-integrin-mediated upregulation of type I collagen synthesis in primary HSC culture (Fig. 7B). We did not observe any difference in LIP-to-LAP ratio in fibronectin-stimulated cells (mean ± SD plastic 1.382 ± 0.348, fibronectin 1.543 ± 0.417; n = 11; P = 0.337).

α5-Integrin gene silencing with siRNA in primary cultures of HSC resulted in significant decrease in type I collagen as well as decrease in C/EBPβ. Effectiveness of α5-integrin gene silencing was demonstrated by significant reduction of α5-integrin protein in HSC transfected with siRNA compared with control cultures (Fig. 7C).

To investigate the role of C/EBPβ in type I collagen transcription, we extracted HSC from C/EBPβ−/− mice and wild-type littermates. HSC were isolated and plated on plastic and fibronectin-coated wells as described above. When plated on plastic there was no detectable difference in morphology or proliferation of HSC isolated from C/EBPβ−/− with wild-type littermates (data not shown). Immunofluorescence for αSMA was positive on day 6 HSC cultures isolated from C/EBPβ−/− animals. The pattern of αSMA staining was similar to that seen in cells from wild-type littermates (Fig. 8A, I and 2). Type I collagen was reduced in the C/EBPβ−/− derived HSC compared with wild-type; fibronectin matrix did not induce more type I collagen synthesis (Fig. 8B).

The levels of α5-integrin on day 6 did not differ in HSC isolated from C/EBPβ−/− animals compared with the wild type. However, when cells were plated on fibronectin, levels of α5-integrin relative to β-actin were higher in the wild-type HSC but failed to increase in HSC from C/EBPβ−/− animals (Fig. 8C). Therefore C/EBPβ plays a role not only in baseline expression of type I collagen genes but also in fibronectin induced changes in α5β1-integrin synthesis. We described above that fibronectin induces type I collagen...
transcriptional control machinery directing changes in ECM during the early activation of HSC.

DISCUSSION

Activation of HSC by liver injury of any type results in transition from a quiescent phenotype to a highly proliferative, matrix-synthesizing cell type. In the case of chronic injury, the synthesis in early activated HSC through $\alpha_5\beta_1$-integrin.

HSC of C/EBP$\beta$ $^{-/-}$ genotype failed to increase $\alpha_5$-integrin levels when stimulated by fibronectin. Therefore C/EBP$\beta$ is a likely regulator of both type I collagen and $\alpha_5$-integrin transcription and serves as part of complex

![Fig. 3. Increase in chloramphenicol acetyltransferase (CAT) activity (representing COL1A1 expression), COL1A1 mRNA, and type I collagen protein in the growth media during primary culture of HSC. HSC were isolated from transgenic mice livers and plated as described in MATERIALS AND METHODS. Cultures were fed daily for the duration of experiment and harvested at designated time points. A: CAT activity increases as HSC activation progresses. CAT was extracted at days 3, 5, 7, 8, 16, 23, and 28 of the primary culture by harvesting cells followed by 3 cycles of freezing and thawing. CAT activity was determined by incorporation of $[^3]$Hacetate from acetyl CoA into chloramphenicol as described in MATERIALS AND METHODS. The figure is a result of 3 independent experiments with samples from each of independent cultures done in duplicates. There is a significant increase in CAT activity at day 5 and day 7 compared with day 3 ($P < 0.05$). Day 7 CAT activity was also higher than that of day 5 ($P < 0.05$). After day 7 there is a small decrease in CAT activity; however, CAT activity essentially plateaued after day 7. The small decrease in CAT activity after day 7 may be explained by the fact that cells at day 7 are confluent and following time points contain significant amount of cellular debris, suggesting some cell death in very confluent cultures. B: real-time PCR analysis of type I collagen COL1A1 mRNA in the primary cultures of HSC at days 4, 6, and 8 compared with the expression at day 2 (quiescent HSC). Values are expressed as a fold change compared with levels measured in quiescent HSC. COL1A1 mRNA increases as the activation progresses, with the highest levels observed in late primary cultures. C: Western blot analysis of the growth media harvested from primary HSC cultures 3 and 7 days in culture. Media was harvested and concentrated through Ultracell YM-10 centrifugal filter unit and TCA precipitated as detailed in MATERIALS AND METHODS. An equal amount of protein was loaded on 7.5% SDS polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with antibody against type I collagen (SC-8788). There is no significant type I collagen signal in day 3 cultures representing mostly quiescent HSC. In contrast, type I collagen is easily detected in media harvested from day 7 cultures.

![Fig. 4. Dynamic changes of the levels of $\alpha\beta_1$-integrin protein, $\alpha\sigma$-integrin mRNA, and $\alpha\beta_1$-integrin protein in activating stellate cells. This figure shows a representative result of all experiments that were done in triplicates. The common pattern found in all experiments is increasing levels of $\alpha\beta_1$-integrin as well as $\alpha\sigma$-integrin in early primary HSC cultures and decreased levels in passaged HSC and late (past day 7) primary cultures. A: Western blot analysis of HSC extracted at days 2, 3, 4, 5, 6, 7, and 9 of primary cultures, as well as tertiary (Tert) HSC cultures. Total proteins were extracted as detailed in MATERIALS AND METHODS. Total protein (40 $\mu$g) was loaded per lane on 7.5% SDS polyacrylamide gel, transferred to PVDF membrane, and probed with antibody against $\alpha\beta_1$-integrin (Chemicon, Temecula CA). There is an increase in $\alpha\beta_1$-integrin signal as cells activate in primary culture (gradual increase in signal intensity from day 2 to day 7). In contrast, $\alpha\beta_1$-integrin protein levels were reduced at day 9 of primary culture and in passaged HSC cultures. B: real-time PCR analysis of type $\alpha\sigma$-integrin mRNA in the primary cultures of HSC at days 4, 6, and 8 compared with the expression at day 2 (quiescent HSC). Values are expressed as a fold change compared with levels measured in quiescent HSC. $\alpha\sigma$-Integrin mRNA increases as the activation progresses in early primary cultures of HSC. The highest levels were observed at day 6. By day 8 expression of $\alpha\sigma$-integrin returns to the levels similar to one observed in quiescent HSC (day 2). C: Western blot analysis of HSC extracted at days 3, 5, 7 and 9 of primary cultures, as well as tertiary HSC cultures. Total proteins were extracted as detailed in MATERIALS AND METHODS. Total protein (40 $\mu$g) was loaded per lane on 7.5% SDS polyacrylamide gel, transferred to PVDF membrane, and probed with antibody against $\alpha\sigma$-integrin kindly provided by Dr. Bingcheng Wang, MetroHealth Medical Center, Cleveland, OH. As seen with $\alpha\beta_1$-integrin, there is an increase in $\alpha\sigma$-integrin signal as cells activate in primary culture (gradual increase in signal intensity from day 3 to day 7). $\alpha\sigma$-Integrin levels were reduced in day 9 primary cultures and passaged HSC cultures.}
Echistatin concentrations (100 nM, 10 nM and 1 nM) or with primary culture. At 3 days after passage cells were incubated with different HSC cultures. Primary HSC cultures were passaged at confluency (day 5).

Changes in CAT activity are shown as percentage changes compared with the control values (CTRL). There is a dose-dependent inhibition of CAT activity compared with control cells plated on plastic (*P < 0.05). Figure represents compilation of 3 independent experiments, each done on 3 samples. B: passaged HSC cultures plated on fibronectin did not show any significant difference in CAT activity compared with control HSC plated on plastic. Figure represents compilation of 3 independent experiments, each done on 3 samples.

result of this transformation is the development of liver fibrosis and ultimately cirrhosis. Not only does the amount of ECM increase severalfold in cirrhosis, the composition of the ECM matrix also changes. Low-density basement membrane-type matrix rich in type IV collagen is replaced by fibrillar type I collagen that is synthetized de novo by activated HSC. Activation of HSC is a dynamic process; however, sequential changes in transcriptional regulation leading to the fully activated phenotype are not fully understood. Because type I collagen is ultimately responsible for development of fibrosis in the liver, defining the differences in regulatory mechanisms controlling its synthesis during the different stages of HSC activation is a preface to any therapeutic intervention.

Our data indicate that different regulatory mechanisms are involved in the expression of type I collagen by HSC in a fashion that is dependent on the phase of HSC activation. Type I collagen was downregulated by α5β1-integrin blocking antibodies and an α5β1-integrin inhibitor in a dose-dependent manner only in primary HSC cultures. α5β1-Integrin inhibitors were ineffective in decreasing type I collagen in passaged HSC, even though passaged HSC continue to synthesize large amount of type I collagen. In addition, fibronectin, the natural α5β1-integrin ligand, increased the COL1A1 transgene activity only in primary HSC. CAT transgene activity was not affected by fibronectin in passaged HSC. These data support the observation that α5β1-integrin-mediated regulation of type I collagen is operative only in early in activation process.

Fibronectin is released very early in liver injury by nonstellate cells, as well as synthesized by stellate cells.
siRNA showed reduced expression of COL1A1 and hypothesized that, as HSC activate, fibronectin binds to the α5β1-integrin and initiates signaling that results in upregulation of type I collagen gene transcription. In the later stages of activation, α5β1-integrin presence on the membrane is downregulated and HSC utilize different mechanism(s) for continued expression of high levels of type I collagen.

At the present time it is unclear what mechanism(s) are responsible for this continued type I collagen synthesis. This switch in type I collagen regulation may be, at least in part, the result of changed composition of the ECM surrounding the HSC. Previous studies have shown that DDR2, which binds type I collagen, is upregulated in activated HSC and its stimulation contributes to proliferation and invasiveness of activated HSC (32). In addition, type I collagen- and α5-integrin-mediated signaling may affect transcriptional regulation of type I collagen genes by upregulation of a transcriptional enhancer. α5β1-Integrin as well as other integrins have been reported to modify activity of transcription factors in certain cell types (18, 19). One such transcription factor reported to play a role in regulation of type I collagen transcription in HSC is C/EBPβ. C/EBPβ is a member of the leucine zipper family of CCAAT enhancer-binding proteins. All members of this family are important regulators of gene expression, particularly involved in inflammatory response and energy metabolism (44, 36). C/EBPβ is present in the majority of cells in two main isoforms. The isoform p35 (LAP) contains both DNA binding and activating domains, whereas the p20 isoform (LIP) contains only DNA binding domain. The p20 isoform does not contain an activating domain and therefore acts as a natural dominant negative isoform, binding to the cis-element on gene promoters but lacking transcription enhancement ability. Both isoforms are translated from a single mRNA molecule, using the same reading frame but different translation start sites (29). The ratio of p35 to p20 has been reported to be instrumental in the transcriptional regulation of αSMA, modulation of cell cycle during liver regeneration, as well as in PEPC expression in hyperglycemic conditions in the liver (14, 25, 37). In HSC, C/EBPβ has been reported to mediate acetaldehyde- and hydrogen peroxide-induced upregulation of type I collagen tran-
transferred, and probed with antibodies against mature type I collagen (SC-8788, Santa Cruz Biotechnology). HSC isolated from C/EBP
plastic and fibronectin.

of primary culture HSC were replated on slides. Cells were incubated on slides for 3 more days to achieve full activation and than fixed with 4% formaldehyde.

Fig. 8. Analysis of HSC isolated from C/EBP
-H9252/H11002

previous studies (16) documented that activated HSC nuclear extracts contain

Recent data using overexpression of C/EBP
 dominant negative isoform in bone of the transgenic animals showed a
decrease in bone formation and reduced COL1A1 expression,
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 on type I collagen
transcription may be similar in all type I collagen-producing

Our experiments suggest that fibronectin increases
C/EBPβ levels in primary HSC cultures, without consistent
changes in the p35-to-p20 ratio. It appears that C/EBPβ
enhances both baseline and fibronectin-stimulated type I
collagen transcription. HSC isolated from C/EBPβ knockout
mice showed lower baseline type I collagen synthesis as
well as blunted response to fibronectin coating. C/EBPβ
knockout also abolished fibronectin-stimulated increase in
α5-integrin, suggesting that ligand-induced upregulation of
α5β1-integrin may in part be mediated by C/EBPβ. The
CCAT/enhancer protein binding site has been previously
identified in the α5-integrin promoter sequence and was
reported to act as a transcriptional enhancer in keratinocytes
during wound healing (5). Baseline expression of α5-integrin
appears to be C/EBPβ independent; its presence on
membrane of HSC isolated from knockout animals did not
differ from wild-type littermates. This dual action of
C/EBPβ on the transcription of both collagen and α5-integrin
genes likely has a synergistic effect on the final
amount of type I collagen synthesized and secreted by early
activated HSC.

In conclusion, the results of this study suggest that type I
collagen expression is regulated by activation phase-specific
mechanisms. Differences in type I collagen control in the early
and late phases of HSC activation might be particularly rele-
vant since both animal and human data indicate that early
fibrosis may be reversible. This study suggests that α5β1-
integrin upregulates type I collagen in primary HSC cultures.
C/EBPβ transcription factor may be instrumental in mediating
this α5β1-integrin effect, although details of this mechanism
remain to be elucidated. Passaged HSC utilize a different
mechanism to maintain high levels of type I collagen synthesis.
Understanding this differences in regulation of type I collagen

scription (2). In addition, the p20 isoform has been shown to
decrease type I collagen gene transcription in HSC in response
to TNF-α treatment (15). Binding sites for C/EBP proteins
exist throughout the type I collagen gene(s) promoter se-
quence. In particular, the sequence between –365 and –335 of
murine α1(I) collagen promoter appears to play a role in
acetaldehyde-induced and C/EBPβ-mediated stimulation of
type I collagen transcription (11). A previously reported analysis
suggested that activated HSC nuclear extracts contain
mainly C/EBPβ isoform, with other C/EBP proteins being
present in much smaller amounts (2).

Recent data using overexpression of C/EBPβ dominant
negative isoform in bone of the transgenic animals showed a
decrease in bone formation and reduced COL1A1 expression,
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gene(s) transcription may be important in designing new therapeu- tic strategies for the management of different stages of liver fibrosis.

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