Impaired Proteolysis of Collagen I Inhibits Proliferation of Hepatic Stellate Cells

IMPLICATIONS FOR REGULATION OF LIVER FIBROSIS*

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Myofibroblastic-activated hepatic stellate cells are the major source of the collagen I-rich extracellular matrix in liver fibrosis but also produce matrix metalloproteinases, which remodel this protein. We have investigated the role of collagen I proteolysis in both regulating proliferation and maintaining the activated myofibroblastic phenotype of stellate cells in vitro. Compared with stellate cells plated on normal collagen I, those plated on a collagenase-resistant form of collagen I (r/r collagen) had reduced thymidine incorporation and proliferating cell nuclear antigen expression but increased p21 expression. Collagen I was shown to be rendered resistant to matrix metalloproteinases by artificial cross-linking in vitro using tissue transglutaminase exerted similar antiproliferative effects on stellate cells to r/r collagen. Of the stellate cell activation markers examined (tissue inhibitor of metalloproteinases-1, α-smooth muscle actin, metalloproteinases-2 and -9, and procollagen I) only the last was decreased by culture on r/r collagen relative to normal collagen I. Antagonists of integrin αvβ3, an integrin reported to stimulate stellate cell proliferation, significantly inhibited adhesion, proliferation, and procollagen I synthesis of stellate cells plated on normal collagen I but had reduced effectiveness on these parameters in cells on r/r collagen. We conclude that proliferation of stellate cells is promoted by pericellular collagen I proteolysis acting via αvβ3 integrin. Cross-linking of collagen I by tissue transglutaminase, a process known to occur in chronic liver fibrosis, might not only increase its resistance to matrix metalloproteinases thereby inhibiting resolution of fibrosis but also functions to constrain the fibroproliferative process.

During liver fibrogenesis profound changes occur in the normal liver extracellular matrix (ECM) most notably an accumulation of interstitial collagens type I and III. It is well recognized that perisinusoidal hepatic stellate cells (HSCs), which become activated to an α-smooth muscle actin (α-SMA)-positive myofibroblastic phenotype following liver injury, are major producers of this neomatrix of fibrosis (1). In vitro studies suggest that the accumulation of interstitial collagens affects the proliferation, survival, and biosynthetic activities of liver cells, including endothelial cells, hepatocytes, and HSCs (2–5). There is increasing awareness that key facets of HSC biology are regulated by their pericellular ECM. For example, collagen I, the major component of fibrosis, enhances the proliferation of HSC in vitro via signaling through the type II discoidin domain receptor (6). In contrast, transfer of activated HSCs onto a basement membrane like matrix (Matrigel) inhibits HSC proliferation and their production of fibrogenic proteins (5). Integrins are key regulators of cellular responses to ECM. We have recently reported that αvβ3 integrin regulates HSC in culture, promoting HSC proliferation while also protecting the cells against apoptosis after serum deprivation (7). Therefore, ligands for αvβ3, which are known to accumulate around HSCs in injured liver, e.g. fibronecgin, may facilitate fibroproliferation in vivo (8, 9). Activated HSCs also produce matrix metalloproteinases (MMPs), including MMP-1, -2, -13, and -14 (10), which may remodel the abundant pericellular collagen I deposited around these cells in vivo to reveal epitopes for αvβ3 integrin. Expression of MMP-2 and -14 is raised persistently in injured and fibrosing liver with HSCs being a key producer (11–15). Remodeling of pericellular collagen by these MMPs may influence key facets of HSC biology: for example our previous studies show that blockade of MMP-2 expression in cultured HSCs using an antisense oligonucleotide effectively inhibited their proliferation (12). In contrast, factors that protect ECM against remodeling might act as a brake on HSC proliferation. Once secreted, fibrotic ECM can be subject to a maturation process in which covalent intermolecular cross-links are incorporated by the enzyme tissue transglutaminase (tTG), which forms covalent cross-links between glutamine and lysine residues. These e-(γ-glutamyl) lysine isopeptide cross-links are resistant to mammalian proteases and increase the strength and insolubility of tissue transglutaminase; HBSS, Hanks’ buffered salt solution; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; WT, wild type; TIMP-1, tissue inhibitor of metalloproteinase-1; C t, threshold cycle; PCNA, proliferating cell nuclear antigen; r/r, collagenase-resistant form of collagen I; TAMRA, tetramethylrhodamine.
ity of ECM as well as its resistance to MMP degradation (16, 17). tTG protein and enzyme activity becomes increased in chronically fibrosed tissues with ECM extracted from cirrhotic liver being demonstrably cross-linked by tTG (18–21). This cross-linking might compromise the resolution of fibrosis. Indeed, our studies have shown a negative correlation between the extent of tTG cross-linking within liver scars and the capacity of the liver to recover from fibrosis (19). Rats administered CCl4 for 12 weeks develop dense fibrosis with tTG cross-links clearly evident in the more mature collagen septa. After a prolonged (12 month) recovery period after CCl4 was virtually complete within just 28 days after CCl4 dosing was stopped. Cross-linking of ECM therefore might stabilize it against remodeling by MMPs, which is a requirement for resolution of fibrosis. However, the consequences of this ECM stabilization for the HSCs embedded within it remain uncertain.

In this study, we have used cell culture models to examine the responses of activated HSCs to culture on both a mutant form of MMP-resistant collagen I (r/r collagen) (22) and collagen I made resistant to MMPs by artificial cross-linking with tTG in vitro. We show that both these matrices maintain HSC activation but inhibit their proliferation. Furthermore, we show evidence that non-degradable collagen exerts these effects through reduced capacity to ligate integrin avβ3, an integrin that we have previously described as a mediator of HSC proliferation.

EXPERIMENTAL PROCEDURES

All reagents were from Sigma-Aldrich unless otherwise stated.

Experimental Models of Progressive Fibrosis and Fibrosis Resolution—Experimental models of reversible liver fibrosis and partly resolving cirrhosis were established as described (19) by injecting cohorts of Sprague-Dawley rats with CCl4 twice weekly intraperitoneally for 6 or 12 weeks, respectively. For each model, livers were harvested at peak fibrosis (3 days after weekly intraperitoneal dosing) and, for the 12 week CCl4 model only, at 365 days after the final dose of CCl4. All procedures were licensed by the United Kingdom Home Office.

In Situ Proteolysis of Liver Matrix by MMP-2—These studies examined whether fibrous septa in cirrhotic rat liver became inherently more resistant to MMP degradation. Cryostat sections of 10 μm were prepared from rat livers harvested at the following times: (a) 12-week CCl4 peak fibrosis liver; (b) liver at 365 days after final 12-week dose of CCl4; (c) 6-week CCl4 peak fibrosis liver. Sections were washed three times in Hanks’ buffered salt solution (HBSS, Invitrogen) containing Ca2+ (HBSS plus Ca2+). Activated form of MMP-2 (Calbiochem) was diluted to 9 μg/ml in HBSS plus Ca2+ and 44 μl added to sections. Control sections were incubated with HBSS plus Ca2+ only. Sections were incubated in a humidified chamber for 8 h at 37 °C. After three washes in phosphate-buffered saline, interstitial collagens remaining undigested in the liver septa were stained with 0.2% Sirius red in saturated picric acid for 2 h. Four low power fields of cryostat liver sections treated with or without active form of MMP-2 for 8 h at 37 °C were stained with Sirius red and subject to computerized image analysis to quantitate area of staining. Following background subtraction (staining away from the collagen septa), the red pixel counts were calculated.

Similarly, rat hepatic stellate cells—Human HSCs were extracted from the margins of normal human liver resected for colonic metastatic disease and purified using density gradients as described (24). Local ethics committee approval was obtained for these studies. Rat HSCs were extracted from normal rat liver using Pronase and collagenase as described (12). HSCs were purified to 90–95% purity by density gradient centrifugation (human HSCs) followed by counter-current elutriation (rat HSCs). HSCs were cultured on plastic in DMEM/FCS for 10 days to induce their conversion to α-SMA-positive myofibroblastic cells then passaged using trypsin/EDTA as described. Cells were used for studies after 1–3 passages.

Isolation of Human and Rat Hepatic Stellate Cells—Human HSCs were extracted from the margins of normal human liver resected for colonic metastatic disease and purified using density gradients as described (24). Local ethics committee approval was obtained for these studies. Rat HSCs were extracted from normal rat liver using Pronase and collagenase as described (12). HSCs were purified to 90–95% purity by density gradient centrifugation (human HSCs) followed by counter-current elutriation (rat HSCs). HSCs were cultured on plastic in DMEM/FCS for 10 days to induce their conversion to α-SMA-positive myofibroblastic cells then passaged using trypsin/EDTA. The HSCs were used for studies after 1–3 passages.

Preparation of tTG Cross-linked Collagen I—Acid-soluble collagenase-resistant (r/r) collagen was extracted and purified from the tails of the mutant mice as described (25). The purity of the final preparation was verified by SDS-PAGE and Coomassie Blue staining.

Preparation of tTG Cross-linked Collagen I—Acid-soluble collagen I from rat tail, hereafter referred to as wild-type (WT) collagen, was diluted to 2 mg/ml in 0.05 M Na2CO3/NaHCO3, pH 9.7. One milliliter of diluted collagen was mixed with 100 μl of cross-link reaction buffer (Tris·HCl, 50 mM, pH 8, containing 2.5 mM CaCl2). After adding guinea pig liver tTG at 0, 50, or 100 ng/ml final concentration, pH was adjusted to 7.5 and cross-linking left to proceed at 30 °C for 6 h. The formation of ε(γ-glutamyl)lysine peptide cross-linked products was detected by Western blotting of reaction products with antibody raised against this cross-link (19). Sources of all immunostaining antibodies are shown in Table 1. To heat denature rat tail collagen I, the acetic acid-solubilized tail collagen was neutralized with 0.2 M NaOH, and the mixture was heated to 60 °C for 30 min.

Preparation of 3H-Labeled Collagen I and tTG Collagen I and MMP Degradation Assay—Acid-soluble collagen I extracted from rat tails as above was radiolabeled using [3H]acetic anhydride (Amersham Biosciences) (25). The 3H-labeled collagen was diluted with unlabeled collagen to obtain a specific activity of 30 cpm/μl (5 μg/μl, 600 cpm/μl). 1 ml of radiolabeled collagen was mixed with 100 μl of cross-link reaction buffer, adjusting final pH to 7.5 and left at 30 °C for 6 h. To assess MMP degradation of the collagens, equal cpm of untreated WT collagen and tTG cross-linked collagens were treated with 20
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Equal amounts of cell protein equivalents were applied to each lane of 4–12% gradient gels or 10% Novex Nu-PAGE gels (Invitrogen). Samples were electrotransferred onto polyvinylidene difluoride, and immunodetection was performed using the primary antibodies and horseradish peroxidase-labeled secondary antibodies shown in Table 1. No bands were detected in negative control blots probed using non-immune IgG instead of primary antibodies. Reactive bands were identified using an enhanced chemiluminescence kit (Amersham Biosciences) followed by autoradiography according to the manufacturer’s instructions.

**TABLE 1**

Details of the antibodies, suppliers, and working concentrations used in this study

| Primary antibodies | Secondary antibodies |
|--------------------|----------------------|
| e(γ-Glutamyl)lysine, 1:200, Chemicon | Goat anti-mouse IgM, 1:2,000, Chemicon |
| tTG, 1:500 | Goat anti-mouse, 1:5,000, Sigma |
| Integrin αv, 1:1,000, Santa Cruz Biotechnology | Goat anti-rabbit, 1:5,000, Sigma |
| Integrin β3, 1:1,000, Santa Cruz Biotechnology | Goat anti-rabbit, 1:5,000, Sigma |
| P21, 1:1,000, Santa Cruz Biotechnology | Goat anti-rabbit, 1:3,000, Sigma |
| PCNA, 1:500, Santa Cruz Biotechnology | Goat anti-rabbit, 1:3,000, Sigma |
| Integrin αvβ3, 1:1,000, Santa Cruz Biotechnology | Goat anti-mouse, 1:3,000, Sigma |
| Biotechnology | Biotechnology |
| β-Actin, 1:500, Sigma | Goat anti-mouse, 1:10,000, Sigma |
| α-SMA, 1:500, Sigma | Goat anti-mouse, 1:10,000, Sigma |

ng/ml of activated forms of MMP-1 (Calbiochem) or MMP-2 (Calbiochem) for 2 h at 30 °C in Tris-HCl, 50 mM, pH 8, containing 2.5 mM CaCl₂. After precipitation with 1 ml of ice-cold 10% trichloroacetic acid and centrifugation at 10,000 × g for 5 min, soluble collagen peptides in 300 μl of the supernatant were measured using a beta counter. The “blank” value of unincorporated ³H present in the trichloroacetic acid supernatant of normal or tTG collagen before treatment with or without MMP-2 was subtracted from all treatments. The cpm of ³H released into trichloroacetic acid supernatants was expressed as percentage of total collagen cpm added into samples.

**Studies of HSCs Cultured on WT, r/r, or tTG Cross-linked Collagen**—Either 24-well or 6-well tissue culture plates or 25-cm² tissue culture flasks, as appropriate, were coated with these collagens at a concentration of 50 μg/cm² at 4 °C overnight. Coated plasticware were washed three times with HBSS. Bovine serum albumin (0.5%) in DMEM was added to plates for 1 h at 4 °C to block uncoated plastic. Activated HSCs previously cultured on plastic were trypsinized and replated for 16 h on the coated plates at a density of ~3 × 10⁶ cells/ml in DMEM plus 16% FCS. For assessment of cell proliferation, HSCs cultured on the different collagen matrices or gelatin in 24-well plates were incubated with serum-free DMEM for 24 h, and then the cells were exposed to DMEM plus 16% FCS for 24 h in the presence of echistatin or neutralizing antibodies to integrins (Table 1) at the concentrations shown. [³H]Thymidine (0.5 μCi/well) was added during the final 18 h of incubation. Thymidine incorporation was assessed as described (12). Results were calculated as cpm/μg of DNA in the cell monolayer as described (5) to compensate for potentially differing numbers of cells adhering to the WT, r/r, and tTG collagen or gelatin matrices at the outset of the studies.

In studies of the role of αvβ3 integrin in HSC adhesion, the disintegrin echistatin (100 nM) was mixed with suspensions of activated HSCs for 30 min before seeding the cells for 2 h on WT or r/r collagen. Non-adherent cells were removed by two gentle washes with HBSS. Adherent cells were stained with Methylene blue as described (5).

**Western Blotting**—HSCs were removed from culture plates with a rubber scraper and lysed by 3× freeze-thawing in phosphate-buffered saline, and aliquots were taken for protein quantitation using the bicinchoninic acid assay. 2× SDS sample buffer was added to the lysate, and proteins were subjected to SDS-PAGE in reduced or non-reduced condition as stated.
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media were replaced by DMEM containing 0.5% FCS. Counting of cells by microscopy showed that equal numbers of cells were present on WT and t/r collagen plates. Cells were left untreated or were stimulated with 4 μM recombinant active form of MMP-2 for 8 h, then [3H]thymidine (1 μCi/well) was added, and cells were incubated for a further 16 h. All treatments were performed in triplicate. [3H]Thymidine incorporation into DNA was assessed as detailed (12).

Statistical Analysis—Significance of differences between means was evaluated using Student’s t-test. Values of $p < 0.05$ were considered significant. Unless otherwise stated, studies were repeated at least three times.

RESULTS

The Collagenous Septa of Cirrhotic Liver Show Resistance to MMP Degradation—It has been reported that tTG cross-linking of purified collagen increases its resistance to MMP-mediated degradation (16, 17). In support of this, we have previously reported that tTG cross-links were present in fibrous septa within cirrhotic livers of rats after 12 weeks dosing with CCl4 and in the abundant septa remaining in the livers even 1 year of recovery (19). In contrast, negligible cross-linking occurred in septa in the livers of rats dosed for only 6 weeks with CCl4 and in the abundant septa remaining in the livers even 1 year of recovery (19). In contrast, negligible cross-linking occurred in septa in the livers of rats dosed for only 6 weeks with CCl4 and in the abundant septa remaining in the livers even 1 year of recovery (19). We therefore examined if cross-linked collagenous septa differed from non-cross-linked in susceptibility to MMP-2 added directly to cryostat sections of rat liver (in situ degradation assay). MMP-2-treated sections from 6-week CCl4-treated rats, when subsequently stained with Sirius red to detect fibrillar collagen, showed that collagenous septa were effectively degraded (Fig. 1). In contrast, identical treatment of cryostat sections of liver from cirrhotic 12-week CCl4-treated rats and from rats allowed to recover for 1 year after such CCl4 treatment, were highly resistant to incubation with MMP-2 as the extent of Sirius red staining of septa was similar both before and after MMP-2 treatment (Fig. 1). Quantitation of the Sirius red staining in multiple fields by image analysis gave the following results: 6-week CCl4 without MMP, 9,831 units; plus MMP-2, 2119 units; 12-week CCl4 without MMP, 27,880 units; plus MMP-2, 15,021 units; 12-week CCl4 plus 1 year recovery without MMP, 11,900 units; and plus MMP-2, 9,728 units. Therefore, MMP-2 treatment resulted in a loss of 79% of Sirius red staining in 6-week CCl4 samples, 46% of staining in 12-week CCl4 samples, but only 18% of staining in 12-week CCl4 plus 1 year recovery samples.

Collagen I Cross-linked by tTG in Vitro Resists MMP-mediated Degradation—Purified collagen I was radiolabeled with [3H]acetic anhydride, and then an aliquot of this collagen I was cross-linked in vitro by incubation with purified tTG. The effectiveness of cross-linking was assessed by monitoring the incorporation of $\epsilon$-(γ-glutamyl)lysine cross-links into collagen products by Western blotting (Fig. 2A). The incorporation peaked at 100 ng/ml tTG enzyme, and therefore this concentration was used to prepare $\epsilon$-(γ-glutamyl)lysine cross-linked collagen (tTG collagen). The [3H]-labeled WT and tTG collagen were treated with active forms of MMP-1 and MMP-2. Following trichloroacetic acid precipitation of high molecular weight undegraded collagen, radiolabel present in degraded collagen peptides (soluble in trichloroacetic acid) was quantified. As shown in Fig. 2B, tTG treatment of collagen increased its resistance to degradation by both MMPs.

HSCs Cultured on Collagen I Cross-linked with tTG Show Reduced Proliferation—Studies were performed to examine whether HSCs cultured on MMP-resistant tTG cross-linked collagen had altered proliferation. Human HSCs were replated onto WT collagen or tTG collagen, and proliferation was assessed by [3H]thymidine incorporation assay. This revealed that the cells on tTG-collagen had a significant 27% decreased

![Figure 1](image1.png)

![Figure 2](image2.png)
proliferation rate compared with the cells on WT collagen I (Fig. 3). Western blotting of cell homogenates showed that expression of proliferating cell nuclear antigen (PCNA), which is expressed in cells during cell cycling (27), was reduced in homogenates of cells cultured on tTG collagen, whereas expression of a mitosis inhibitor, the cyclin-dependent kinase inhibitor p21, was increased.

**Effect of Non-degradable r/r Collagen on HSCs Proliferation**—Human HSCs activated by culture on tissue culture plastic were trypsinized and replated onto films of either WT rat tail collagen I or collagen I extracted from the tails of r/r mutant mice, and proliferation on the different collagens was assessed by [3H]thymidine incorporation assay. Cells on r/r collagen had proliferation significantly reduced by 40% compared with HSCs plated on WT collagen (Fig. 4A). Western blotting showed expression of PCNA was also reduced in HSCs on r/r collagen (Fig. 4B). In contrast, synthesis of the cyclin-dependent kinase inhibitor p21 showed an opposite pattern, being increased in cells on r/r collagen.

In complementary studies, proliferation was examined in mouse HSCs extracted from the livers of normal or r/r collagen mutant mice and then plated onto plastic. These activated HSCs produce an r/r collagen I-rich matrix that is deposited onto culture plates, thus providing a further model of r/r versus WT collagen culture substrata. These studies confirmed those with human HSCs above, showing that proliferative response to FCS was significantly decreased by 61% in the r/r HSCs compared with WT HSCs (Fig. 4C).
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HSCs on Non-degradable Collagen I Show Reduced Proliferative Response to MMP-2—We have previously reported that inhibiting MMP-2 expression by HSCs by treatment with antisense DNA inhibited their proliferation, suggesting that MMP-2 is an autocrine mitogen for HSCs in culture (12). As well as being a gelatinase with activity against degraded interstitial collagen, MMP-2 has been shown to degrade intact collagen I (28). We therefore examined whether proliferation of HSCs in response to added active MMP-2 required the degradation of pericellular collagen I. Passaged human HSCs were plated onto either WT collagen or r/r collagen and treated for 24 h with 4 nM of the active form of MMP-2. When [3H]thymidine incorporation was assessed, cells on WT collagen showed a 125 ± 12% (p < 0.003) increase in proliferation in response to MMP-2 (Fig. 5). However, proliferative response to MMP-2 was negligible in MMP-2-treated cells plated on r/r collagen.

Effect of Non-degradable Collagen I on Activation of HSCs—Activation of HSCs in culture is influenced by ECM composition. For example, in our previous studies transfer of activated HSCs from plastic to a basement membrane-like matrix (Matrigel) profoundly reduced their expression of procollagen I, \( \alpha \)-SMA, and TIMP-1 (5). Therefore, the influence of WT and r/r collagen culture substrata on HSC expression of these activation markers was examined. TaqMan quantitative PCR analysis showed that activated HSCs plated on r/r collagen synthesized 42% less mRNA for procollagen I \( \alpha \)-1 compared with HSCs on WT collagen (Fig. 6A), but TIMP-1 and \( \alpha \)-SMA mRNA expression were not significantly affected. Western blotting showed that expression of the myofibroblastic marker protein \( \alpha \)-SMA was slightly reduced in cells on r/r collagen (Fig. 6B). During activation in culture, HSCs produce a variety of MMPs, including the gelatinases MMP-2 and -9 (12, 29). Secretion of these MMPs by HSCs plated on WT or r/r collagen was examined by gelatin zymography of HSC-conditioned media. Clear zones of gelatin lysis were present corresponding to 72-kDa MMP-2 and 92-kDa MMP-9. However, there was no difference in secretion of these MMPs between cells plated on either form of collagen (Fig. 6C).

\( \alpha \)v\( \beta \)3 Expression and Utilization by HSCs on WT and r/r Collagen—We have previously reported that the integrin heterodimer \( \alpha \)v\( \beta \)3 becomes more highly expressed by HSCs as they undergo activation in culture. Ligation of \( \alpha \)v\( \beta \)3 enhances the proliferation of HSCs in culture, because disintegrins targeting this integrin, e.g. echistatin, have antiproliferative effects (7). In the current study, Western blotting showed that expression of the \( \alpha \)v\( \beta \)3 heterodimer and the individual \( \alpha \) and \( \beta \)3 monomers was differentially regulated by the matrices. Expression was reduced in HSCs plated on r/r collagen, relative to WT collagen (Fig. 7). \( \alpha \)v\( \beta \)3 integrin reportedly has a low affinity for intact collagen I, being more effective in mediating binding of cells to denatured collagen I. Activated HSCs produce a number of MMPs with interstitial collagenase activity, including MMP-2 and MMP-14 (28, 30), which might remodel pericellu-
lar collagen I to reveal cryptic RGD binding sites for \( \alpha_\beta_3 \) integrin (31, 32). However, \( r/r \) collagen would be resistant to these MMPs. We therefore examined whether \( \alpha_\beta_3 \)-dependent adhesion might differ in HSCs plated on WT versus \( r/r \) collagen. In cultures of HSCs added to WT collagen, the \( \alpha_\beta_3 \) disintegrin echistatin significantly blocked cell adhesion (Fig. 8). However, echistatin had no effect on adhesion of HSCs to \( r/r \) collagen, suggesting that \( \alpha_\beta_3 \) integrin plays little role in HSC adhesion to this substrate. We previously reported that optimum proliferation of activated HSCs in response to serum required \( \alpha_\beta_3 \) ligation (7). As stated above, this integrin binds preferentially to degraded rather than intact collagen. We therefore examined the extent to which this integrin stimulated proliferation on WT versus \( r/r \) collagen. In HSCs plated on WT collagen I, echistatin and neutralizing antibodies to \( \beta_3 \) integrin significantly blocked cell adhesion (Fig. 8). However, echistatin had no effect on adhesion of HSCs to \( r/r \) collagen, suggesting that \( \alpha_\beta_3 \) integrin plays little role in HSC adhesion to this substrate. We previously reported that optimum proliferation of activated HSCs in response to serum required \( \alpha_\beta_3 \) ligation (7). As stated above, this integrin binds preferentially to degraded rather than intact collagen. We therefore examined the extent to which this integrin stimulated proliferation on WT versus \( r/r \) collagen. In HSCs plated on WT collagen I, echistatin and neutralizing antibodies to \( \beta_3 \) integrin significantly inhibited HSC proliferation by 65 and 68%, respectively (Fig. 9A). Neutralizing antibodies to \( \beta_3 \) integrin (20 \( \mu G/ml \)) or 100 \( nM \) echistatin were added during the last 24 h. \(^{3}\text{H}\text{Thymidine} \) incorporation over the last 18 h was measured. Results show mean ± S.E. of three independent studies. **, \( p < 0.001 \) relative to control. B, effects of collagen denaturation on HSC proliferation. Activated rat HSCs were plated onto plasticware plates coated with either WT collagen or heat-denatured WT collagen (gelatin) for 48 h. Cell proliferation was then assessed by \(^{3}\text{H}\text{Thymidine} \) incorporation assay as described. Incorporation into cells on WT collagen was designated 100%. Results show mean ± S.E. of three independent studies. *, \( p < 0.05 \) relative to WT.

**Effect of Integrin Antagonists on Collagen I Expression**—Our current studies suggest that \( \alpha_\beta_3 \) integrin ligation supports...
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![Collagen Regulation of Hepatic Stellate Cells](image)

**FIGURE 10. Effects of αvβ3 antagonists on HSC production of procollagen I mRNA on WT and r/r collagen.** Rat HSCs at passages 1–2 were trypsinized then replated onto plasticware plates coated with WT collagen I (W.T.) or r/r collagen I (r/r) for 48 h. Neutralizing antibodies to β3 integrin (20 μg/ml) or 100 nM echistatin were present during the last 24 h. Expression of procollagen I mRNA was measured by TaqMan quantitative PCR. Data are expressed as the percentage of expression of mRNA by the HSCs left untreated with disintegrins on each coating. Results show mean ± S.E. of four independent studies. *, p < 0.001 relative to control (ctrl).

Type I procollagen expression by HSCs, because both echistatin and neutralizing antibodies against β3 integrin effectively decreased the expression of collagen 1α1 mRNA by cells cultured on WT collagen (Fig. 10). However, these disintegrins less effectively inhibited procollagen I mRNA synthesis by cells plated on non-degradable r/r collagen. This suggests that αvβ3-dependent support of type I procollagen synthesis requires pericellular collagen degradation.

**DISCUSSION**

Liver fibrosis is a dynamic process involving not only net accumulation of ECM but also its ongoing remodeling by proteases, including MMPs (10, 11, 15). Proliferation, survival, and biosynthetic activity of cells are regulated by pericellular matrix, and proteolytic remodeling of matrix can profoundly modify its effector properties (33). Our previous studies in mutant mice expressing r/r collagen showed that collagen I degradation promoted the regenerative response of hepatocytes during resolution of liver fibrosis (34). Hepatocytes plated on r/r collagen had reduced proliferative response to serum compared with cells on native collagen. Integrin αvβ3 contributed to the proliferative response to native collagen, because its blockade reduced hepatocyte proliferation on this matrix without affecting proliferation on r/r collagen. Intact collagen I is known to be a poor ligand for αvβ3 integrin, whereas degradation of collagen I reveals RGD epitopes recognized by this integrin (31, 32). Our studies suggest this mechanism operates in our HSC cultures. Firstly, HSCs showed increased proliferation when plated on heat-denatured type I collagen (gelatin) in which there is exposure of RGD epitopes relative to untreated collagen. Secondly, although untreated HSCs adhered to r/r and WT collagen with similar efficiency, αvβ3 integrin antagonists decreased HSCs adhesion to WT collagen more effectively than adhesion to r/r collagen. The integrin antagonists had similar effects on cell proliferation. These studies suggest that αvβ3-dependent proliferation is only effective if an appropriate form of ligand is present. Our studies also show that HSCs plated on r/r collagen have reduced expression of αvβ3, which might lead to this integrin having a reduced role in adhesion and proliferation on this substrate.

Apart from its effects on proliferation, culture on r/r collagen did not affect expression of the studied HSC activation markers such as TIMP-1, MMP-2, and MMP-9. However, cells on r/r collagen showed significantly reduced expression of procollagen I mRNA. As with proliferation, a role for αvβ3 in supporting procollagen I synthesis on WT collagen, but not on r/r collagen, was evident from the inhibitory effects of αvβ3 antagonists.

Although a pericellular environment of mutant r/r collagen is clearly artificial, we suggest that our studies using this matrix provide insights into effects of biologically relevant mechanisms of collagen stabilization against degradation. We and others have demonstrated tTG cross-linking of fibrotic matrix occurs in advanced liver fibrosis (16, 18), and cross-linking has also been described in other tissues undergoing fibrosis, including kidney (16, 17). Potential sources of tTG in injured liver are hepatocytes (39) and activated HSCs (40). Although this cross-linking is considered to stabilize matrix against degradation, there is little direct evidence to support this, and even less is known about how cross-linking of collagen I affects cellular responses. Our findings indicate that tTG cross-linking of matrix confers resistance to MMPs. Firstly, the collagenous septa in cryostat sections of cirrhotic rat liver, which we previously showed contained tTG cross-links (19), resisted degrada-
tion by exogenous MMP-2 while the septa in liver sections from rats with moderate fibrosis lacking cross-links were readily removed by MMP-2. Secondly, our preparations of collagen I cross-linked by tTG in vitro developed resistance to MMPs. Therefore, tTG cross-linking of collagen I in fibrotic liver might have similar consequences to the mutations in r/r collagen, functioning to inhibit the resolution of fibrosis (34). Our studies are the first to examine the consequences for HSCs of being in contact with MMP-resistant type I collagen. Culture of activated HSCs on tTG collagen or r/r collagen showed clear parallels in that cells on both matrices had reduced proliferation and PCNA expression but increased p21 expression.

We suggest that the acquisition of MMP resistance in ECM in chronic fibrosis might function to limit the cellularity of the liver scar. A wide variety of matrix- and cytokine-mediated mechanisms have been identified that promote HSC activation and proliferation in the context of fibrotic injury (41, 42). However, HSC proliferation in fibrotic liver remains controlled despite the persistence of these mechanisms. Our studies are the first to show that development of MMP resistance in collagen I may act as an antiproliferative stimulus to limit HSC replication. Thus cross-linking as part of scar maturation may act as a brake on the fibroproliferative process. This might represent an example of how chronic and long-lasting changes to the matrix may program a reduced proliferative response within the key ECM-producing cells: the activated HSCs. We suggest that collagen cross-linking not only renders the scar more permanent but also serves to inhibit the cellular facet of the scar response.

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