Activation of Hepatic Stellate Cells by TGFα and Collagen Type I Is Mediated by Oxidative Stress Through c-myb Expression

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Abstract

Excessive production of collagen type I is a major contributor to hepatic fibrosis. Activated (myofibroblastic), but not quiescent, hepatic stellate cells (lipocytes) have a high level of collagen type I and α-smooth muscle actin expression. Therefore, stellate cell activation is a critical step in hepatic fibrosis. Here we show that quiescent stellate cells were activated by the generation of free radicals with ascorbate/FeSO₄ and by malondialdehyde, a product of lipid peroxidation. In addition, stellate cell activation by collagen type I matrix and TGFα was blocked by antioxidants, such as d-α-tocopherol and butylated hydroxytoluene. Moreover, oxidative stress, TGFα and collagen type I markedly stimulated stellate cell entry into S-phase, NFkB activity and c-*myb* expression, which were prevented by antioxidants. c-*myb* antisense oligonucleotide blocked the activation and proliferation of stellate cells induced by TGFα. Nuclear extracts from activated, but not from quiescent, stellate cells formed a complex with the critical promoter E box of the α-smooth muscle actin gene, which was disrupted by c-myb and NFkB65 antibodies, and competed by c-*myb* and NFkB cognate DNA. c-*Myb* expression was also stimulated in activated stellate cells in carbon tetrachloride-induced hepatic injury and fibrogenesis. This study indicates that oxidative stress plays an essential role, through the induction of c-*myb* and NFkB, on stellate cell activation. (*J. Clin. Invest.* 1995. 96:2461–2468.) Key words: c-*myb* expression • stellate cell gene expression • oxidative stress • lipid peroxidation • liver fibrogenesis

Introduction

Collagen type I, is the most excessive extracellular matrix protein in hepatic fibrosis (1, 2), which is in turn a major contributor to the morbidity and mortality of patients affected by chronic liver diseases. Therefore, the regulation of type I collagen gene expression is crucial in understanding the pathogenesis of this disease (3).

Although the mechanisms responsible are unknown, the increased production of collagen α_1(I) in liver injury is mainly a result of an enhanced collagen gene transcription (4, 5). From several lines of investigation, we have obtained evidence indi-

cating that aldehyde-protein adducts, including products of lipid peroxidation, modulate collagen gene expression (6–8) and may be a link between tissue injury and fibrosis (9–11). Whether the basis of fibrogenesis in disorders associated with lipid peroxidation, such as in iron overload (12), chronic viral hepatitis (Lee et al., unpublished observations), porphyria (13), alcohol- (14), and carbon tetrachloride-induced (15) hepatic injury, is linked to this intriguing process remains to be elucidated. However, we demonstrated a temporal relationship as well as colocalization of lipid peroxidation and increased expression of collagen α_1(I) gene, in the liver of rats treated with carbon tetrachloride or overloaded with iron (10, 11). These results suggest that lipid peroxidation affects collagen α_1(I) transcription directly and/or through the activation of cells responsible for its synthesis.

In this context, stellate cells play a key role in the pathogenesis of hepatic fibrosis (16, 17). Although we (18) and others (19, 20) reported that quiescent stellate cells produce little collagen type I, activated (myofibroblastic) stellate cells display a high level of collagen α_1(I) gene expression (16, 17, 19). Therefore, stellate cell activation is a critical step in hepatic fibrogenesis. Studies with primary cultures of adult rat stellate cells have provided evidence that cell-type specific growth regulatory mechanisms exist (21), but the stellate cell-specific factors regulating stellate cell proliferation and activation have not yet been identified.

In this study, we analyzed a mechanism leading to stellate cell activation. We found that oxidative stress is a common and indispensable step, in the cascade of molecular events initiated by collagen type I matrix or TGFα, resulting in stellate cell activation and stellate cell proliferation. Moreover, our results suggest that the activation of NFkB and the induction of c-*myb* nuclear expression by oxidative stress, are critical for the activation of stellate cells.

Methods

Cell cultures. Stellate cells were prepared from male Sprague-Dawley (400–500 grams) by in situ perfusion and single-step density Nycodenz gradient (Accurate Chemical & Scientific Corp., Westbury, NY), as described previously (10, 18). The cells were mixed with 9.5 ml Hank's containing 3 grams/liter bovine serum albumin and 8 ml of 28.7% (wt/vol) Nycodenz in sodium-free Hank's buffer. The gradient was generated by placing 6 ml of the Hank's/albumin solution on top of the liver cell mixture in a 50-ml centrifugation tube. After centrifugation (1,000 *g*, 4°C, 20 min) the cells were aspirated from above the interface, washed twice in serum-free DME regular glucose medium and subsequently cultured under an atmosphere of 5% CO_2, 95% air in tissue culture dishes using DME medium containing penicillin G 100 U/ml, streptomycin sulfate 100 µg/ml and 10% fetal calf serum. For TGFα-induced stellate cell activation, cells were cultured on plastic in a serum-free defined media (Fibroblast basal medium with Insulin, Clonetics). Fibroblast basal medium is similar to F12 medium, but contains folinic acid, Hepes buffer with NaOH, $MgSO_4$ and adenine instead of folic acid, $NaHCO_3$, $MgCl_2$ and hypoxanthine. Cells were plated on 60-mm

dish coated with collagen type I, EHS matrix (Matrigel) or plastic (according to the experimental design), for the initial seeding of fat-storing cells at a density of $2-3 \times 10^6$. Matrigel's (Collaborative Biomedical Products) major components are laminin, collagen IV, proteoglycans, entactin and nidogen. It also contains $TGF\beta$, fibroblast growth factor, and tissue plasminogen activator. Treatments were started 18 h after hepatic stellate cell isolation, and continued for additional 120 h for cells cultured on plastic (with serum), EHS or collagen I matrices, and for additional 60 h for cells cultured in serum-free defined media. Medium was changed every 24 h for all conditions. The sequences of c-myb oligonucleotide phosphorothioate were: sense (5' GCC CGG AGA CCC CGA CAC 3') and antisense (5' GTG TCG GGG TCT CCG GGC 3'). Stellate cells were identified by their typical autofluorescence at 328 nm excitation wavelength, staining of lipid droplets by oil red, and immunohistochemistry with a monoclonal antibody against desmin (10).

Nuclear extract preparation. Nuclei were prepared by a modification of the procedure described previously (22, 23). Cells were homogenized in 1 ml of 5% citric acid, 0.5% NP-40, 10 mM NaF and 10 mM Na pyrophosphate with a glass Dounce homogenizer with a loose fitting pestle. The homogenized cells were placed above a cushion consisting of 30% sucrose and 1% citric acid. The nuclei were precipitated by a 4,000 g centrifugation at 4°C for 30 min and frozen at -70°C. Gel retardation analysis of protein–DNA complexes were performed with an oligonucleotide of the putative DNA binding site, as described previously (23, 24). The sense oligonucleotide were: NFkB (5' GGG GAC TTT CCC 3') and α-smooth muscle actin E box (5' GAT CAT AAG CAG CTG AAC TGC C 3').

Animals. C57BL/6 mice (20–25 grams) each received a single intraperitoneal injection of CCl_4 in mineral oil (1:3, vol/vol) at a dose of 2 ml/kg body weight, or mineral oil only (control). After 48 h, animals were sacrificed and liver tissues were promptly removed, fixed in 10% formaldehyde and embedded in paraffin for immunohistochemical staining (10).

Immunohistochemistry. Cells, fixed with acetone: methanol (50:50) at -20°C for 20 min, and liver tissue were immunostained as described previously (11, 23). Antibodies directed against c-myb, NFkB65, α-smooth muscle actin, desmin, or proliferating cell nuclear antigen (PCNA)[1] were obtained from Sigma Chemical Co. (St. Louis, MO), 5 Prime 3 Prime, and Oncogene Sciences. Fluorescent labels were visualized using a dual channel Zeiss microscope as described previously (23). A phase-contrast microscope was utilized to visualize antigens with alkaline phosphatase secondary antibodies (Vector Laboratories). Cytochromes utilized were alkaline phosphatase with fast green as counterstain, and FITC with Evans blue as counterstain (Sigma Chemical Co.). The number of PCNA(+), c-myb(+), or NFkB(+), α-smooth muscle actin (SMA)(+) cells was expressed as a percentage of total cells. At least 1,000 cells are analyzed per each experimental point, and a minimum of two observers analyzed each immunohistochemical experiment independently as described previously (23). Negative control samples were processed in parallel under the same conditions, but with omission of the first antibody.

Statistical analysis. Results were expressed as mean of at least triplicates unless stated otherwise. Either the Student t or the Fisher's exact test (two-tailed) was used to evaluate the differences of the means between groups, with a P value of < 0.05 as significant.

Results

First, we examined the role of lipid peroxidation on stellate cell activation. Stellate cell activation was induced in quiescent cells growing on a EHS matrix, while inhibition of this phenotype was attempted in cells activated by collagen type I matrix (19, 20). When stellate cell activation was induced by TGFα (21),

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1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; PCNA, proliferating cell nuclear antigen; SMA, smooth muscle actin.

a defined media without serum was used. Stellate cell activation was assessed by the expression of α-smooth muscle actin (α-SMA) (25), and S-phase by the presence of proliferating cell nuclear antigen (PCNA) (26).

As depicted in Fig. 1, we found that quiescent stellate cells, cultured on a EHS matrix (*open bars*) (control), were activated by the generation of free radicals using ascorbic acid (200 μM)/FeSO$_4$ (50 μM) as described previously (7). Because we reported that ascorbic acid/FeSO$_4$ induces lipid peroxidation in cultured fibroblasts, with the production of malondialdehyde and 4-hydroxynonenal (7), we tested whether malondialdehyde would mimic the effects of inducing lipid peroxidation. Indeed, malondialdehyde (200 μM) markedly stimulated the activation of hepatic stellate cells (Fig. 1). Malondialdehyde at lower concentrations (50 μM) was also able to activate stellate cells but to a lesser extent (data not shown). As expected, stellate cells cultured on a collagen type I matrix (*closed bars*) or treated with TGFα (12 nM) (*hatched bars*) became activated at a much higher rate than their respective control conditions (Fig. 1). The values of the control condition for TFGα were comparable to those of the EHS control (< 10%). Similarly, stellate cells cultured on plastic and treated with malondialdehyde displayed a more activated pattern than cells grown on plastic (93 vs. 58% of cells (+) for α-SMA; $P < 0.05$). Complementary results supporting the role of oxidative stress on stellate cell activation, included the findings that induction of stellate cell activation by a collagen type I matrix can be blocked by antioxidants, such as d-α-tocopherol (50 μM) or butylated hydroxytoluene BHT (50 μM) (Fig. 1). In addition, d-α-tocopherol blocked the activation of stellate cells on plastic (14 vs 58% of cells (+) for α-SMA; $P < 0.05$). In Fig. 2, representative examples of the association between oxidative stress and stellate cell activation, assessed by α-SMA immunohistochemistry, are shown. The expression of α-SMA (indicated in *brown*) was markedly induced in stellate cells treated with malondialdehyde (Fig. 2 *B*) when compared to control stellate cells cultured on a EHS matrix (Fig. 2 *A*). A similar stimulation of α-SMA expression was observed when stellate cells were cultured on collagen type I (Fig. 2 *C*), in agreement with previous reports (19, 20). Interestingly, this increased expression of α-SMA was abolished by d-α-tocopherol (Fig. 2 *D*), or BHT (Fig. 1). A small percentage of the cells (< 5%) displayed mild to moderate staining when the first antibody was omitted.

Control stellate cells on a EHS matrix (Fig. 3) or control TGFα stellate cells were quiescent in regard to proliferation, with only $\sim$ 5% of the cells in S-phase. In contrast, oxidative stress induced by ascorbic acid/FeSO$_4$, collagen type I matrix, and TGFα markedly increased stellate cell proliferation, judging by the percentage of cells in S-phase (> 66% for all conditions). Malondialdehyde, a product of lipid peroxidation, also stimulated stellate cell entry into S-phase. In addition, antioxidants such as d-α-tocopherol or BHT, blocked the activation of the stellate cell cycle induced by collagen type I matrix or TGFα. Representative examples of the nuclear expression of PCNA (*in brown*), an index of the cell cycle S-phase (26), are shown in Fig. 4. It was negligible in control stellate cells (Fig. 4 *A*) or when the first antibody was omitted, but it was dramatically induced in stellate cells exposed to oxidative stress (Fig. 4 *B*).

Because NFkB plays important roles in the regulation of cell growth and function, and oxidative stress increases NFkB activity (27, 28), we analyzed the potential role of NFkB regulation in stellate cell activation. Stellate cell activation was asso-
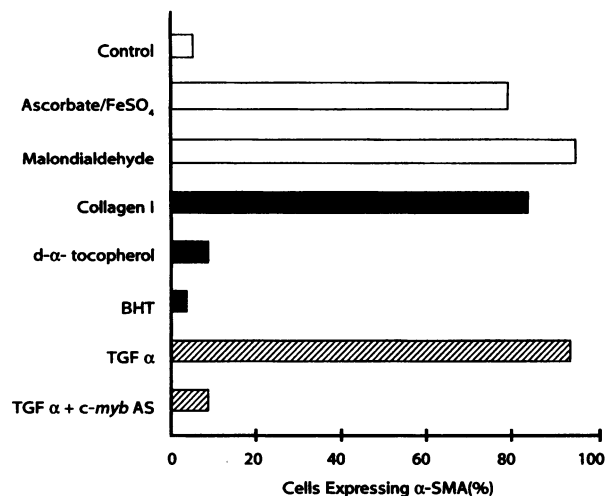
Figure 1. Oxidative stress induces the expression of α-smooth muscle actin in hepatic stellate cells. α-Smooth muscle actin (α-SMA) expression was detected by immunohistochemistry in primary stellate cells. Cells were cultured on EHS matrix (*open bars*) (control, ascorbic acid [200 μM]/FeSO₄ [50 μM], and 200 μM malondialdehyde), collagen type I matrix [closed bars] (collagen I, 50 μM d-α-tocopherol, and 50 μM butylated hydroxytoluene [*BHT*]), or plastic (*hatched bars*) (12 nM TGFα with and without 25 μM c-myb antisense [*AS*]). Cells on EHS or collagen type I were cultured in media with 10% fetal calf serum, while cells on plastic were cultured on serum-free media. Values represent the percentage of cells positive for α-SMA; $P < 0.05$ for ascorbic acid/FeSO₄, malondialdehyde, collagen and TGFα. The SEM was < 30% of the mean for all conditions.

Figure 3. Oxidative stress induces hepatic stellate cell proliferation. The nuclear expression of PCNA was detected by immunohistochemistry in primary stellate cells. Cells were cultured on EHS matrix (control, ascorbic acid [200 μM]/FeSO₄ [50 μM], and 200 μM malondialdehyde), collagen I matrix (collagen I, 50 μM d-α-tocopherol and 50 μM BHT), or plastic (12 nM TGFα with and without 25 μM c-myb antisense [AS]). Cells were cultured as described in Fig. 1. Values represent the percentage of cells positive for PCNA; $P < 0.05$ for ascorbic acid/FeSO₄, malondialdehyde, collagen, and TGFα. The SEM was < 30% of the mean for all conditions.

ciated with the nuclear translocation and activation of NFkB, as detected by immunofluorescence (Figs. 5 and 6) and gel shift analysis (Fig. 7). As shown in Fig. 5, the nuclear expression of NFkB as detected with specific antibodies against NFkB65, was

substantially increased in stellate cells cultured on a EHS matrix and treated with malondialdehyde, or when cultured on a collagen type I matrix. The lipophilic antioxidant d-α-tocopherol blocked the activation of NFkB induced by collagen type I, indicating that collagen type I affects NFkB activation through oxidative stress. Representative examples of NFkB expression are shown in Fig. 6. Quiescent stellate cells lacked nuclear NFkB (Fig. 6 A), whereas stellate cells activated with malondialdehyde displayed nuclear expression of NFkB (Fig. 6 B). Negligible background nuclear fluorescence was observed when the first antibody was omitted. As depicted in Fig. 7, the binding of stellate cells nuclear extracts to a NFkB cognate oligonucleotide was low in quiescent cells (Fig. 7, lane 2), but increased significantly following stellate cell activation (Fig. 7, lane 3).
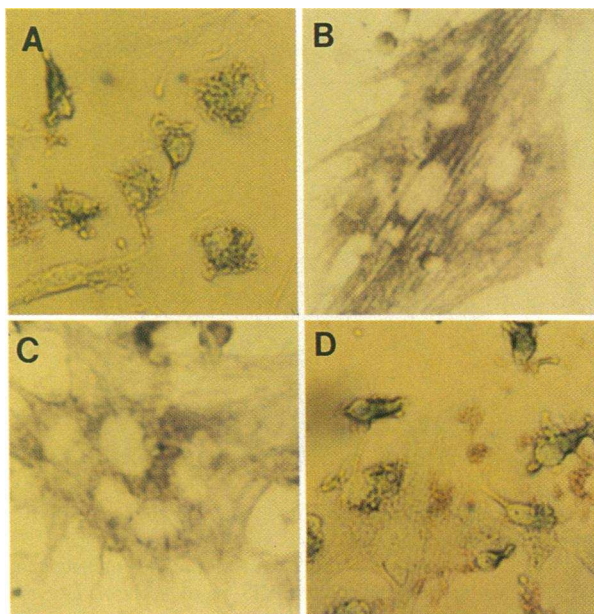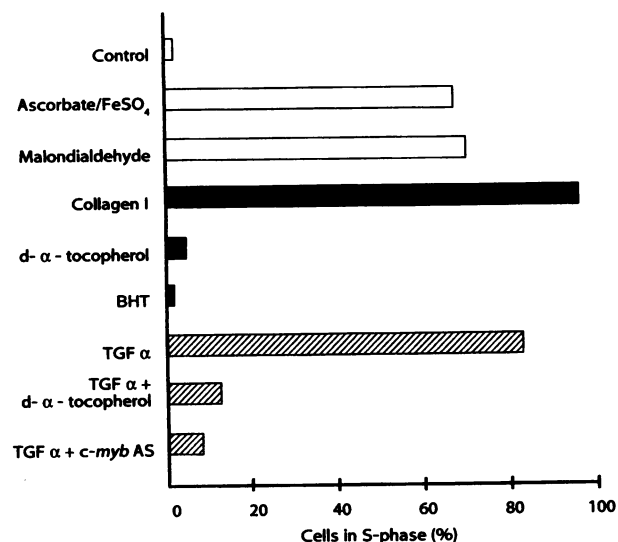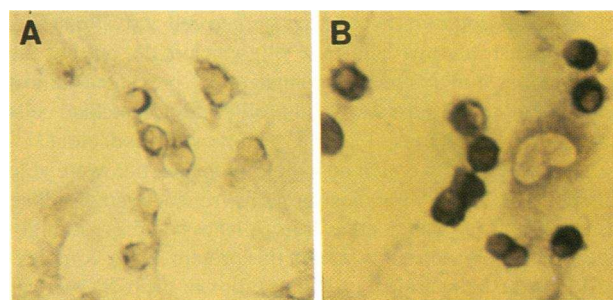

Figure 2. Representative examples of the association of α-smooth muscle expression and oxidative stress in hepatic stellate cells. α-SMA expression was detected using alkaline phosphatase immunohistochemistry (brown), while fast green counterstained all cells. αSMA immunohistochemistry is shown for control (A), 200 μM malondialdehyde (B), collagen type I matrix (C), and 50 μM d-α-tocopherol (D). ×100.

Figure 4. PCNA expression and oxidative stress are associated in hepatic stellate cells. PCNA was detected using alkaline phosphatase immunohistochemistry (brown), while fast green counterstained all cells. PCNA immunohistochemistry is shown for cells cultured on EHS matrix (control) (A) and for ascorbic acid [200 μM]/FeSO₄[50 μM] (B) ×100.
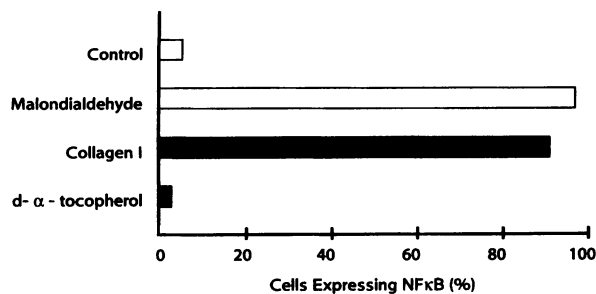
Figure 5. NFkB nuclear expression is increased in activated stellate cells. NFkB nuclear expression was detected by immunofluorescence in primary stellate cells. Cells were cultured on EHS matrix (control and 200 μM malondialdehyde), or collagen type I matrix (collagen I and 50 μM d-α-tocopherol). Cells were cultured as described in Fig. 1. Values represent the percentage of cells positive for nuclear NFkB; P < 0.05 for malondialdehyde and collagen. The SEM was < 15% of the mean for all conditions.

As expected, the binding of nuclear extracts from activated stellate cells to the NFkB site was disrupted by antibodies against NFkB65 (Fig. 7, lane 4), but not by preimmune serum (not shown). In addition, the NFkB oligonucleotide abolished the complex of ^{32}P-labeled NFkB oligonucleotide and nuclear extracts from activated stellate cells (Fig. 7, lane 6). In brief, antioxidants prevented stellate cell activation (Figs. 1 and 3), and NFkB nuclear activity (Fig. 5 and 7) induced by ascorbic acid/FeSO$_4$, collagen type I or TGFα.

Because c-myb is an important inducer of proliferation in hematopoietic and smooth muscle cells (29–31), we tested whether c-myb expression was important for stellate cell proliferation and/or activation. First, we found that the expression of c-myb, detected by immunofluorescence using monoclonal c-myb antibodies, was enhanced in activated stellate cells, irrespective of the method of induction, and that this effect was prevented by antioxidants (Fig. 8). The nuclear expression of c-myb was increased by the generation of oxidative stress with ascorbic acid/FeSO$_4$, malondialdehyde, collagen type I matrix, and TGFα. Next, we assessed whether c-myb expression affected stellate cell activation. TGFα induction of stellate cell activation was chosen for the c-myb antisense experiments because the control stellate cells were uniformly quiescent in defined media and in the absence of serum. Less than 5% of control cells for the TGFα experimental condition expressed nuclear c-myb. c-Myb antisense oligonucleotide suppressed the nuclear expression of c-myb during stellate cell activation (Fig. 8). Of interest, a c-myb antisense oligonucleotide, but not the corresponding c-myb sense oligonucleotide, prevented almost completely both the activation and proliferation of stellate cells induced by TGFα (Figs. 1 and 3). Moreover, monoclonal c-myb antibodies and a c-myb cognate oligonucleotide were able to disrupt the binding of nuclear proteins from activated stellate cells to a NFkB oligonucleotide (Fig. 7, lanes 5 and 7), strongly suggesting the presence of c-myb in this protein/DNA complex.

As expected, the critical promoter E box of the α-SMA gene (32), formed complexes with nuclear extracts from activated stellate cells (Fig. 9, lane 3), but not with nuclear extracts from quiescent stellate cells (Fig. 9, lane 2). Relevant to this study, the protein-DNA complexes (b) were markedly disrupted by monoclonal c-myb antibodies (Fig. 9, lane 4) and NFkB65 antibodies (Fig. 9, lane 5), and competed by oligonucleotides
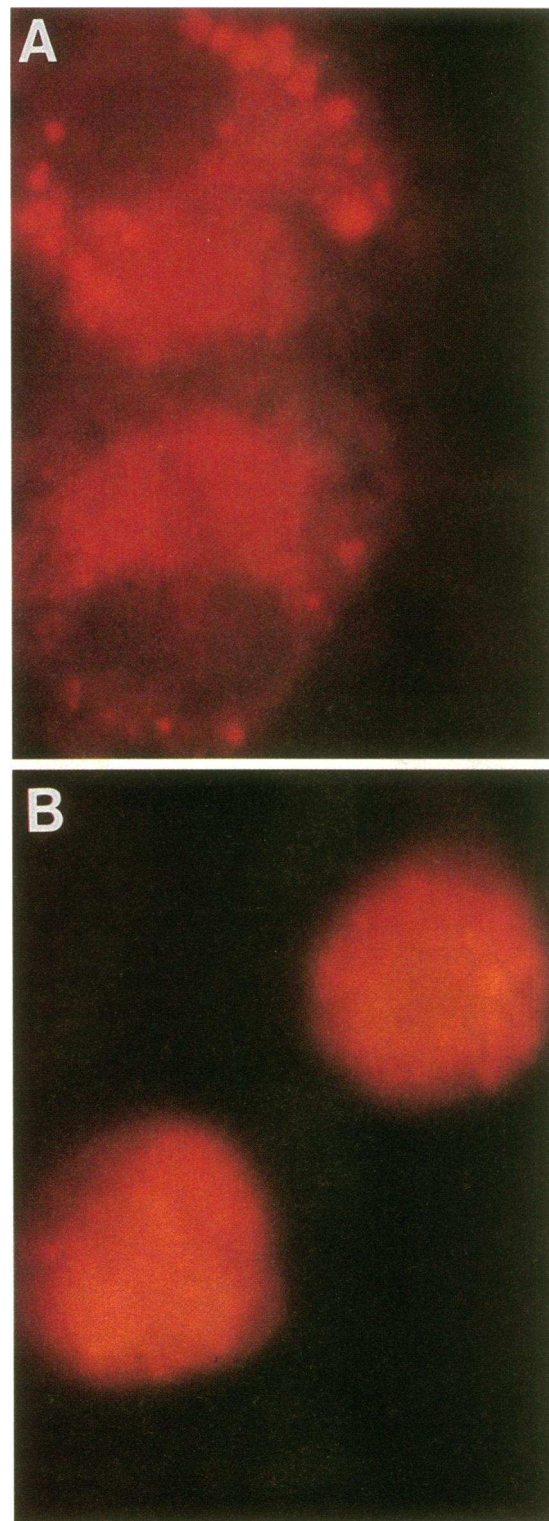

Figure 6. Representative examples of the association between NFkB nuclear expression and oxidative stress. NFkB was detected using NFkB65 and fluorescein-labeled second antibodies. NFkB immunofluorescence is shown for cells cultured on EHS matrix (control) (A) and for 200 μM malondialdehyde (B). ×100.

spanning either the c-myb site or the NFkB site (Fig. 9, lanes 6 and 7). Preimmune serum did not affect the protein-DNA complexes (not shown). As expected, the α-SMA oligonucleo-
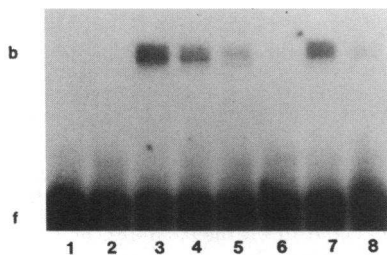
Figure 7. NFkB binding activities are increased in activated stellate cells. Mobility shift analysis of nuclear extracts from quiescent (lane *2*) and activated (lanes *3–8*) stellate cells. Samples were incubated with ^{32}P-labeled NFkB oligonucleotide (1 ng). The DNA-protein complexes were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel. The positions of the free (*f*) and bound (*b*) DNA are indicated. Nuclear extracts on lanes *4* and *5* were preincubated with NFkB65 antibodies and monoclonal c-myb antibodies, respectively. Nuclear extracts on lanes *6, 7,* and *8* were incubated with consensus oligonucleotides (10 ng) for NFkB, c-myb and α-SMA, respectively. Lane *1* was incubated without nuclear extracts.

Figure 9. c-Myb is a major contributor to the α-smooth muscle actin binding activities of activated stellate cells. Mobility shift analysis of nuclear extracts from quiescent (lane *2*) and activated stellate cells (lanes *3–8*) was performed with samples incubated with ^{32}P-labeled α-SMA promoter E-box (1 ng). The DNA-protein complexes were resolved as described in Fig. 7. Nuclear extracts on lanes *4* and *5* were preincubated with monoclonal c-myb antibodies and NFkB65 antibodies, respectively. Nuclear extracts on lanes *6, 7,* and *8* were incubated with consensus oligonucleotides (10 ng) for NFkB, c-myb and α-SMA, respectively. Lane *1* was incubated without nuclear extracts.

tide competed the binding of nuclear extracts from activated stellate cells to the NFkB (Fig. 7, lane *8*) and α-SMA (Fig. 9, lane *8*) oligonucleotides. These results strongly suggest a role for c-myb (and presumably NFkB) on a α-SMA gene expression, a hallmark of stellate cell activation (25).

To ascertain whether these findings in culture stellate cells are relevant to the role of activated stellate cells in hepatic fibrosis, we studied the expression of c-*myb* in an animal model of liver fibrogenesis. Mice were treated with carbon tetrachloride, an hepatotoxin, that induces liver lipid peroxidation (15), malondialdehyde-protein adducts (10), stellate cell activation (25), collagen gene expression (5, 17), and in chronic treatments, liver cirrhosis (5, 33). Moreover, coculture experiments of hepatocytes and stellate cells treated with carbon tetrachloride, indicate that hepatocytes exert a paracrine stimulation of both lipid peroxidation and collagen gene expression on stellate

Figure 8. Oxidative stress induces c-myb expression in hepatic stellate cells. c-myb expression was detected by immunohistochemistry in primary stellate cells. Cells were cultured on EHS matrix (control, ascorbic acid [200 μM]/FeSO$_4$[50 μM], and 200 μM malondialdehyde), collagen type I matrix (collagen I and 50 μM d-α-tocopherol), or plastic (12 nM TGFα alone or with the addition of either 50 μM d-α-tocopherol or 25 μM c-*myb* antisense [AS]), as described in Fig. 1. Values represent the percentage of cells positive for c-myb; $P < 0.05$ for ascorbic acid/FeSO$_4$, malondialdehyde, and collagen I and TGFα. The SEM was < 30% of the mean for all conditions.

cells (10). In this context, immunofluorescence studies established that c-*myb* expression (in *yellow*) was markedly increased in sinusoidal cells, with the phenotype of activated stellate cells, in liver sections of animals treated with carbon tetrachloride (Fig. 10 *B*). Omission of the first antibody was associated with modest and diffuse background fluorescence. We found that c-*myb* expression is negligible in the liver of control animals treated with the vehicle mineral oil (Fig. 10 *A*).
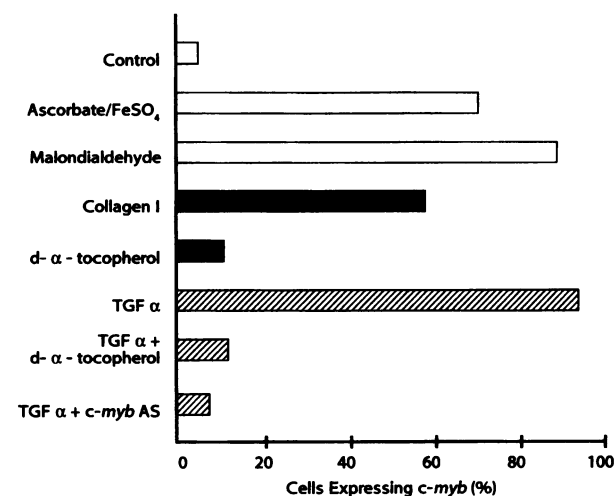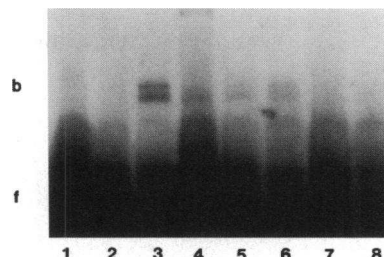
Discussion

Although overproduction of collagen type I by hepatic stellate cells is a critical step in the development of liver cirrhosis (16, 17, 19, 20), the regulation of collagen α_1(I) gene expression remains unclear (3). In this study, we have characterized some of the cellular pathways that are involved in the activation of quiescent stellate cells. Oxidative stress mediates the activation of stellate cells by collagen type I matrix or TGFα. Moreover, we have identified a molecular mechanism leading to stellate cell activation.

Our results suggest a critical role of NFkB and c-*myb* on stellate cell activation given that during stellate cell activation the nuclear expression of NFkB and c-*myb* is increased, and that stellate cell activation, is blocked by antisense, but not by sense, c-*myb*. Also, we determined that in activated stellate cells, NFkB and c-myb contribute substantially to the nuclear binding activities to the promoter E box of the α-SMA gene. These findings strongly suggest that NFkB and c-myb are the molecular mediators of oxidative stress on stellate cell activation, and that they bind to the critical E box of the α-SMA gene (32), the expression of which is intrinsic to the activated phenotype of stellate cells (25). Relevant to this issue, coculture experiments of hepatocytes and stellate cells treated with carbon tetrachloride (a hepatocyte, but not a stellate cell, toxin), indicate that hepatocytes could exert a paracrine stimulation of both lipid peroxidation and collagen gene expression on stellate cells (10). Potential mediators of this model of oxidative stress in stellate cells include free radicals, reactive aldehydes or cytokines produced by the hepatocytes in response to carbon tetrachloride. In support of a role of reactive aldehydes on collagen gene expression, both acetaldehyde (6) and malondialdehyde (7), increase collagen α_1(I) gene transcription, and this effect can be blocked by scavengers of reducing equivalents (7) which are required for the formation of aldehyde-protein adducts. In
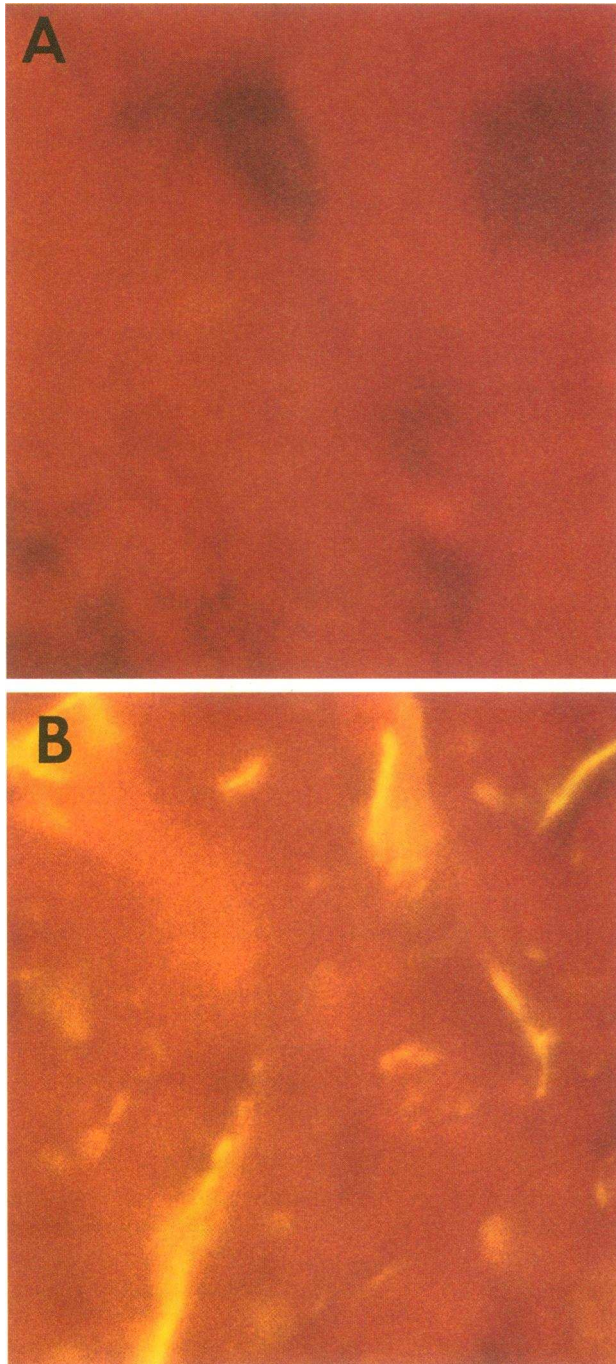
Figure 10. c-myb expression is increased in activated stellate cells in the liver following carbon tetrachloride-induced hepatic injury. c-myb expression (in *yellow*) was detected by immunofluorescence in liver sections using monclonal c-myb and fluorescein-labeled second antibodies, while Evans blue (in *red*) was used as counterstain. Liver sections were obtained from control (*A*) and experimental (*B*) mice, 48 h after the intraperitoneal injection of mineral oil vehicle or carbon tetrachloride in mineral oil, respectively. ×1,000.

agreement with these findings, stellate cell collagen gene expression is stimulated by 4-hydroxynonenal, another product of lipid peroxidation (34).

In this context, we have demonstrated that malondialdehyde or the induction of lipid peroxidation, dramatically activates quiescent stellate cells. Moreover, antioxidants (such as d-α-tocopherol or BHT) suppress the characteristic activation of stellate cells cultured on collagen type I matrix or TGFα. Therefore, oxidative stress is both sufficient and indispensable for the activation of stellate cells.

Furthermore, activated stellate cells may also recruit nonactivated stellate cells by means of a paracrine mechanism, that has been suggested to perpetuate fibrogenesis (35). Several cytokines are known to facilitate activation of stellate cells, including PDGF, FGF, and TGFα (21). Interestingly, we have found evidence to support a convergence of lipid peroxidation and cytokine pathways in the activation of stellate cells. For example, the induction of stellate cell activation and proliferation by TGFα utilizes an oxidative pathway, because can be blocked with antioxidants. In addition, in iron overload in the absence of overt inflammation, lipid peroxidation induces the expression of TGFβ1 and collagen α_1(I) (11). Conversely, TGFβ1 exerts a prooxidant effect on cultured osteoblastic and endothelial cells (36, 37). TGFβ1 (38), lipid peroxidation and its aldehyde products (7), and acetaldehyde (6), a product of ethanol oxidation, which can induce lipid peroxidation (14), are known to stimulate collagen gene transcription. Acetaldehyde increases collagen synthesis in cultured human fibroblasts (39) and in activated stellate cells obtained from baboon liver (40). Also, acetaldehyde stimulates collagen production and mRNA expression in cultured rat stellate cells (41, 42).

Several studies indicate that c-*myb* plays an important role in cell differentiation and proliferation (for review see reference 43). For example, regulation of c-*myb* expression is critical for the growth and differentiation of the progeny of hematopoietic cells (29, 31, 44). c-Myb protein binds to a consensus cognate DNA (45) through three homeo domain-like regions (46), and activates the transcription of target genes (47–49). These genes include the promyelocyte *mim*-1 (50), *cdc*2 (51) c-*myc* (52), and c-*myb* itself (53). A role for c-*myb* affecting the cell cycle before the G1/S phase boundary, has been suggested (54, 55). Our results indicating that c-*myb* expression is important for proliferation of hepatic stellate cells supports its effect on cell entry into S-phase. Similarly, c-*myb* appears to be critical for the proliferation of vascular smooth muscle cells in culture (30) and in an animal model of arterial re-stenosis (56).

Although little is known about the mechanisms that modulate c-*myb* expression, it has been suggested that oxidation of Cys 43 could function as a molecular sensor for the redox state of the cell by affecting the DNA binding affinity of c-myb (57). The modulation of AP1 proteins involving reduction-oxidation is mediated by the nuclear redox factor Ref-1 (58), which also functions as a DNA repair enzyme (27). Ref-1 stimulates DNA binding activity of several transcription factors including c-myb, and may itself be under a posttranslational control which is sensitive to the redox state of the cell (27). The redox activity of Ref-1 is mediated through a conserved cysteine amino acid motif (KCR) that is present in Fos, c-Jun and related proteins. In c-myb, redox changes probably affects the motif KQCR (which includes Cys 43), within the DNA binding domain. In agreement with this novel hypothesis (27, 57), we found that oxidative stress affects the DNA binding activity and expression of c-myb. Although the molecular mechanisms remain to be elucidated, the increased expression of c-*myb* could be achieved for instance, by positive autoregulation of c-*myb* (53) through the oxyredox modulation of c-myb protein (27).

The precise molecular interactions of c-myb with other transcription factors is poorly understood. However, Ness et al. (59) have reported that c-myb and NF-M proteins act as a bipartite activator of myeloid-specific genes. In this context, we

found that LAP(NF-IL6) (22), which is related to NF-M (60), is expressed in hepatic stellate cells (Houglum et al., unpublished observations). Moreover, LAP confers differentiated functions (22, 61–64) and blocks the cell cycle before the G1/S boundary inhibiting the proliferation of hepatoma cells (23). Similarly, LAP is critical for adipocyte differentiation (65), a cell that resembles quiescent hepatic stellate cells in their ability to store fat (19, 65). Collectively, these results suggest the possibility that a LAP/c-myb interaction may occur in stellate cells, affecting the differentiation and/or proliferation of these cells. In addition, stellate cell differentiated functions could be modulated at least in part, by the interaction between LAP and NFkB (66), which is known to affect gene transcription (67). Our study provides insights into the molecular mechanisms leading to hepatic stellate cell activation, as well as a rationale for potential therapeutic approaches for hepatic fibrosis, a major contributor to the morbidity and mortality of patients with chronic liver diseases.

Note added in proof. A recent study by Burgess et al. (68) suggests that the antiproliferative activity of c-*myb* specific oligonucleotides, at least on smooth muscle cells, is not due to a hybridization-dependent antisense mechanism. Rather, a stretch of four contiguous guanosine residues, which is present in the antisense c-*myb* used by us (this publication) and others (30, 56), may be responsible for the sequence-specific but non–antisense antiproliferative effects of these oligonucleotides.

Acknowledgments

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