S-Adenosylmethionine Blocks Collagen I Production by Preventing Transforming Growth Factor-β Induction of the COL1A2 Promoter*

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To study the antifibrogenic mechanisms of S-adenosylmethionine (AdoMet), transgenic mice harboring the −17 kb to +54 bp of the collagen α2 (I) promoter (COL1A2) cloned upstream from the β-gal reporter gene were injected with carbon tetrachloride (CCL4) to induce fibrosis and coadministered either AdoMet or saline. Control groups received AdoMet or mineral oil. AdoMet lowered the pathology in CCL4-treated mice as shown by transaminase levels, hematoxylin and eosin, Masson's trichrome staining, and collagen I expression. β-Galactosidase activity indicated activation of the COL1A2 promoter in stellate cells from CCL4-treated mice and repression of such activation by AdoMet. Lipid peroxidation, transforming growth factor-β (TGFβ) expression, and decreases in glutathione levels were prevented by AdoMet. Incubation of primary stellate cells with AdoMet down-regulated basal and TGFβ-induced collagen I and α-smooth muscle actin proteins. AdoMet metabolites down-regulated collagen I protein and mRNA levels. AdoMet repressed basal and TGFβ-induced reporter activity in stellate cells transfected with COL1A2 promoter deletion constructs. AdoMet blocked TGFβ induction of the −378 bp region of the COL1A2 promoter and prevented the phosphorylation of extracellular signal-regulated kinase 1/2 and the binding of Sp1 to the TGFβ-responsive element. These observations unveil a novel mechanism by which AdoMet could ameliorate liver fibrosis.

S-Adenosylmethionine (AdoMet) is the principal biologic methyl donor, the precursor of aminopropyl groups used in polyamine biosynthesis in liver, and the precursor of reduced glutathione (GSH) through its conversion to cysteine by means of the transsulfuration pathway (1). Under normal conditions, most of the AdoMet generated is used in transmethylation reactions, in which methyl groups are added to compounds and AdoMet is converted to S-adenosylhomocysteine (SAH) (2).

In alcoholic liver disease, many of the enzymatic steps in methionine metabolism are affected (3). In the intragastric infusion model of ethanol feeding, hepatic levels of methionine, AdoMet, and DNA methylation decrease by −40% (4). The hepatic methionine level depends on the stage of liver injury in rats fed ethanol (5). Reduced AdoMet levels and methylation can affect gene expression, membrane fluidity, and GSH levels in liver (2, 6, 7). Rats with decreased hepatic levels of AdoMet are predisposed to liver injury caused by lipoxysaccharide, and this effect is prevented by exogenous AdoMet treatment (8).

AdoMet has been used increasingly for the treatment of liver diseases, although the protective mechanisms remain unclear and are likely to be multiple. Impaired mitochondrial uptake of GSH has been postulated to be an important pathogenic factor in alcoholic liver injury. AdoMet administration restores GSH uptake into mitochondria in rats fed ethanol for 4 weeks by preventing changes in mitochondrial membrane fluidity (7). AdoMet inhibits tumor necrosis factor-α (TNFa) release from macrophages (8), and it has also been used to prevent the development of hepatocarcinogenesis (9) and to increase survival in patients with alcoholic liver disease (10).

Excessive collagen I accumulation is the histopathologic hallmark of liver fibrosis. Central to the development and progression of fibrosis are cytokines that are normally involved in matrix remodeling; among them, transforming growth factor-β (TGFβ) enhances collagen I production and inhibits the synthesis of proteolytic enzymes that catalyze extracellular matrix degradation while enhancing the expression of protease inhibitors (11, 12). As a result, TGFβ is believed to play a critical role in liver fibrosis. There are no data as to whether administration of AdoMet may affect levels of TGFβ itself and/or the actions of TGFβ on collagen I expression and fibrosis.

The current study focused on analyzing potential mechanisms by which administration of AdoMet could decrease collagen I deposition under fibrogenic stimuli. Treatment with AdoMet was found to have a repressive effect on the COL1A2 promoter both in vivo and in vitro. We now propose that these antifibrogenic effects of AdoMet appear to be mediated, at least in part, by lowering TGFβ levels and by inhibiting TGFβ binding to the COL1A2 promoter through a Sp1-pERK1/2 coupled mechanism.

**EXPERIMENTAL PROCEDURES**

Most reagents unless otherwise stated were purchased from Sigma. Protein concentration was determined by the method of Lowry et al. (13) using the DC-20 protein assay kit (Bio-Rad).

**Animal Study Design**—Transgenic mice harboring the −17 kb to +54 bp of the proximal promoter of the mouse COL1A2 gene cloned up-
stream from the *Escherichia coli* β-gal reporter gene (LacZ) were used. These transgenic mice were obtained from Dr. Benoit de Crombrugghe (Department of Molecular Genetics, University of Texas, M. D. Anderson Cancer Center, Houston) (14–18). Mice were bred in our in-house and received humane care in compliance with the guidelines of the National Institutes of Health and the Animal Care Committee of Mount Sinai School of Medicine. Chronic liver injury was induced by injecting intraperitoneally 5 ml of CCl₄/kg of body weight (25% v/v in mineral oil) three times a week for 6 weeks. AdoMet was administered intraperitoneally at a dose of 10 mg/kg body weight every day for 4 days and was always given 2 h before CCl₄. Control groups were injected with mineral oil or mineral oil plus AdoMet alone. Mice were maintained at 22 °C with a 12 h light/dark cycle, fed standard chow diet, had free access to water, and were sacrificed under pentobarbital anesthesia 48 h after the last dose of CCl₄.

**Serum Transaminases—**Blood was collected from the retro-orbital vein of anaesthetized mice killed by cervical dislocation. Serum was separated to assay for alanine aminotransferase (ALT) and aspartate transaminase (AST) using kits from Sigma (Infinity ALT and AST).

**Liver Histology and Immunohistochemistry—**Liver samples were fixed in 10% buffered formalin and embedded in paraffin. Five-μm sections were dehydrated and stained with hematoxylin and eosin (H&E) and Picrosirius red by a liver ethanol bath which was blinded from the experimental conditions. The Sirius red/fast green staining was carried out as described previously (19). Immunofluorescence was carried out using a rabbit IgG fraction to β-gal (ICN Biomedical), the reporter protein for the COL1A2 promoter activation, and a rabbit IgG to α-smooth muscle actin (α-Sma), a specific marker for stellate cell activation. Alexa Fluor 488 dye-conjugated goat anti-rabbit antibody and Alexa Fluor 568 dye-conjugated goat anti-mouse antibody (Molecular Probes) were used for immunofluorescent staining of β-gal (corresponding to the active COL1A2 promoter) and α-Sma, respectively, and colocalization by overlaying both stainings using Adobe Photoshop computer software. TGFβ immunostaining was carried out using a TGFβ monoclonal antibody (Sigma) and a Immunocruz staining system (Millipore). Western blot analysis were performed as described previously (21).

**Cell Culture and Transfection Experiments—**All cell culture experiments were carried out using primary hepatic stellate cells (HSC) isolated by *in situ* liver perfusion and Histodenz gradient centrifugation (27). HSC were cultured in Dulbecco’s modified Eagle’s medium without serum from the beginning of each treatment. The doses of AdoMet, MTA, SAH, and methionine were either 10 or 30 μM. TGFβ was used at 10 ng/ml. Reporter DNA constructs containing upstream sequences of the human COL1A2 promoter linked to the chloramphenicol acetyltransferase (CAT) gene were provided by Dr. Francisco Ramirez (New York Hospital for Special Surgery) (28). In these constructs, human COL1A2 sequences span from −3500 to +58 bp (−3500COL1A2-CAT), from −772 to +58 bp (−772COL1A2-CAT), and from −378 to +58 bp (−378COL1A2-CAT) (28). Parallel transfection of the corresponding empty vector pEMBL5-CAT at equivalent concentrations were performed. The total amount of plasmid DNA was equalized using pBlueScript (Stratagene). Cells were plated at 2×10⁴ cells in 6-well plates. Complexes containing FuGENE 6 (Roche Applied Science) plus plasmid DNA were prepared according to the manufacturer’s instructions with a final concentration of plasmid DNA for each of the chimeric COL1A2 DNA constructs of 1 μg/ml. Parallel cotransfection with 25 ng/ml of the control pRL-null (Promega) containing the cDNA encoding for Renilla luciferase was performed to normalize for transfection efficiency. Cells were incubated in the presence of the transfection mix for 24 h, after which the media were replaced and the cells treated with 30 μM AdoMet for 24 h. Samples for the CAT activity were collected, and the reaction was run using a kit from Promega as described previously (29).

**TGFβ—**TGFβ was measured by enzyme-linked immunosorbent assay using a kit from ELAB Life Sciences.

**Indirect Western Blot Analysis—**Indirect Western blot analysis was performed by the method of Singh et al. (33). The membrane containing the immunoprecipitated pERK1/2 bound to the immunoblotted Sp1 was blocked in 10% nonfat dry milk in TNE buffer (50 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA). DNA binding was carried out for 3 h with TNE buffer containing 5 μg/ml poly[d(I-C)], 150 mM KCl, 0.05 mM ZnCl₂, and 20,000 cpm of labeled probe at room temperature for 30 min. For competition studies, 200-fold of cold probe was added along with labeled probe. PAGE (6%) was performed at 150 V for 2 h in 0.25 × TBE buffer, the gels were dried under vacuum and exposed overnight using Kodak films.

**Southwestern Blot Analysis—**Southwestern blot analysis was performed by the method of Singh et al. (33). The membrane containing the immunoprecipitated pERK1/2 bound to the immunoblotted Sp1 was blocked in 10% nonfat dry milk in TNE buffer (50 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA). DNA binding was carried out for 3 h with TNE buffer containing 5 μg/ml poly[d(I-C)], 2 × 10⁶ cpm/ml [γ-³²P]ATP multiprime-labeled BglII, and BstXI-digested fragment of the −378 COL1A2-CAT plasmid containing the TGFβ-responsive element. Membranes were washed three times for 5 min each with TNE at room temperature and exposed in the PhosphorImager screen.

**Statistical Analysis—**Analyses of variance were performed for all experiments except for those in Fig. 4B, which were analyzed by an unpaired Student’s t test. Values are expressed as the means ± S.E. and are the average values of three and six experiments for the in vitro and in vivo studies, respectively.

**RESULTS**

**Induction of Liver Fibrosis in Mouse COL1A2 Promoter Transgenic Mice and Protection by AdoMet—**Administration of CCl₄ increased the liver to body weight ratio ~15% over the non-CCl₄-treated mice with no amelioration by AdoMet. Hepatocellular damage and protection by AdoMet were assayed by measuring serum levels of transaminases and by H&E staining. The CCl₄ treatment elevated ALT and AST serum levels 7- and 3.5-fold, respectively. AdoMet lowered the elevated ALT and AST levels using a lipid peroxidation colorimetric assay kit (Calbiochem). Catalytic activity of CYP2E1 was determined as the rate of production of p-nitroanisole from p-nitrophenol (25).

**Northern blot analysis was performed** as described previously (21, 26) using cDNA probes for COL1A1 (provided by Dr. Scott L. Friedman, Mount Sinai School of Medicine) and COL1A2 (provided by Dr. Francesco Ramirez, New York Hospital for Special Surgery) and a cDNA for GAPDH, a housekeeping gene, from the ATCC.
levels in the CCl₄-treated mice to control levels (Fig. 1A). H&E staining in the CCl₄-treated mice revealed the presence of Mallory bodies, lymphocyte infiltration, centrilobular steatosis, and perivenular and pericellular fibrosis; AdoMet prevented these changes and modified the pathology (Fig. 1B). Similarly, Masson’s trichrome staining showed less endogenous total collagen deposition in mice treated with CCl₄ plus AdoMet than in the CCl₄-treated mice (Fig. 1C). Quantitative analysis of total collagen in Sirius red- and fast green-stained liver sections showed a 2.5-fold increase by CCl₄, which was prevented by AdoMet treatment (Fig. 1E). Collagen I expression assessed by Western blot analysis was elevated 3-fold by the CCl₄ treatment and reduced by about 50% by coadministration of AdoMet (Fig. 1D).

AdoMet Prevents the Activation of the Mouse COL1A2 Promoter in Vivo—Transgenic mice harboring the −17 kb to +54 bp of the proximal promoter of the mouse COL1A2 gene cloned upstream from the E. coli β-gal LacZ reporter gene have been shown to be valuable in studies of activation of the COL1A2 gene in vivo (14, 16, 18). CCl₄ activated the COL1A2 promoter as shown by the β-gal-positive blue staining (Fig. 2A). This activation was repressed in mice cotreated with AdoMet (Fig. 2A). Quantification of the β-gal activity by chemiluminescence showed an approximate 27-fold increase in β-gal activity by CCl₄, which was reduced to a 5-fold increase in the presence of AdoMet (Fig. 2B). Immunofluorescence analysis of mice injected with CCl₄ revealed colocalization of α-Sma, a marker for stellate cell activation, and β-gal-positive cells, indicating that the activation of the COL1A2 promoter occurred in HSC (Fig. 2D).

AdoMet increased total GSH about 17% in control mice. When CCl₄ was injected, a 15% decrease in GSH levels was observed compared with the mineral oil-injected mice; however, cotreatment with AdoMet restored GSH to above control levels (Fig. 3A). AdoMet decreased lipid peroxidation by-products generated under CCl₄ treatment to the levels found in control mice (Fig. 3B). Immunostaining for 4-hydroxynonenal showed an extensive but diffuse pattern of positive staining, indicating development of oxidant stress in all liver cells (not shown). CYP2E1 expression and activity were lower in the CCl₄-treated mice compared with the control mice because CYP2E1 expression and activity were lower in the CCl₄-treated mice (Fig. 3C). Western blot analysis of HSC lysates showed a clear induction of collagen I protein and mRNA levels by AdoMet (Fig. 3D). These results suggest that the down-regulation of TGFβ production under CCl₄ plus AdoMet treatment in vivo (Fig. 3, A and B) is likely happening in Kupffer cells.

Experiments were next carried out to assess whether AdoMet could prevent the mechanism(s) involved in the TGFβ-mediated stimulation of collagen I expression in HSC. Western blot analysis of HSC lysates showed a clear induction of collagen I expression by TGFβ treatment compared with control cells, and this effect was totally blunted by coadministration of AdoMet 24 h prior to the TGFβ addition (Fig. 6D). These results imply that AdoMet can affect collagen I protein likely by modulating TGFβ levels and actions.

To address whether AdoMet could prevent the TGFβ-mediated transactivation of the human COL1A2 promoter, primary HSC were transfected with the −378COL1A2-CAT construct, which contains the TGFβ-responsive element (−313 to −250 bp from the transcription start site, Fig. 7B), and treated 24 h later with AdoMet. AdoMet blunted the TGFβ transactivation of the COL1A2 promoter (Fig. 7A). Furthermore, gel mobility shift assays using the −378 to −183 bp region of the human COL1A2 promoter, containing the TGFβ-responsive element as a probe, confirmed the increased presence of bound proteins after TGFβ treatment and showed that AdoMet administration prevented the formation of these TGFβ-stimulated DNA complexes (Fig. 7C). These complexes were competed using the cold probe containing the TGFβ-responsive element (Fig. 7C, Comp lane).
FIG. 1. AdoMet reduces CCl₄-induced liver fibrosis. A, the activities of ALT and AST are presented in units/liter as the means ± S.E. (n = 6). **, p < 0.01 for CCl₄ versus MO; ★★★, p < 0.001 for MO + AdoMet versus MO; ★★, p < 0.01 for CCl₄ + AdoMet versus CCl₄; and ★★★★, p < 0.001 for CCl₄ + AdoMet versus MO + AdoMet. B, H&E staining. Mice injected with CCl₄ showed Mallory bodies (*), lymphocyte infiltration (★★), and centrilobular steatosis (★). Administration of AdoMet minimized the pathology caused by CCl₄. (original magnification, ×200). C, collagen...
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FIG. 2. β-Gal staining and quantification. A, activation of the mouse COL1A2 promoter is shown in mice treated with CCl₄ (blue color), whereas repression of such activation is found in mice cotreated with AdoMet (magnification, ×200). B, the activity of β-gal, an indicator of induction of the mouse COL1A2 promoter, was quantified by chemiluminescence, and the results are expressed as AU of chemiluminescence/mg of protein. MO, mineral oil; SAM, S-adenosylmethionine. ●●●, p < 0.001 for CCl₄ + AdoMet versus CCl₄; ****, p < 0.001 for CCl₄ versus MO; and ▲▲▲, p < 0.001 for CCl₄ + AdoMet versus MO + AdoMet. C, mouse COL1A2 promoter scheme showing the enhancer region and the proximal promoter where the TGFβ-responsive element is located. D, immunofluorescence analysis for the mouse COL1A2 promoter and α-Sma was carried out with antibodies against β-gal, the reporter gene for the mouse COL1A2 promoter, and α-Sma, a marker for stellate cell activation. Mice injected with CCl₄ revealed colocalization of α-Sma and β-gal-positive cells, indicating activation of the mouse COL1A2 promoter in HSC (magnification, ×200).

Detection was carried out by the Masson’s trichrome staining. Less collagen deposition (dark color) was observed in mice treated with CCl₄ plus AdoMet than in CCl₄-treated mice where collagen was distributed in the perportal and pericellular areas (magnification, ×200). D, collagen I expression analyzed by Western blot. The numbers under the blot indicate arbitrary units (AU) of densitometry for collagen I corrected by β-tubulin expression. E, for the quantitative analysis of total liver collagen the Sirius red/fast green method was used. Results are given as average values ± S.E. (n = 6) and are expressed in μg of collagen/mg of protein. MO, mineral oil; SAM, S-adenosylmethionine. ●●●, p < 0.001 for CCl₄ + AdoMet versus CCl₄; ****, p < 0.001 for CCl₄ versus MO; and ▲, p < 0.05 for CCl₄ + AdoMet versus MO + AdoMet.
Ramirez and collaborators (11, 12, 32, 38) have located the TGFβ-responsive element of the human COL1A2 gene between nucleotides 313 and 250, relative to the transcription start site. The TGFβ-responsive element sequence consists of two nearly juxtaposed footprints (Boxes 3A and B), the most distal of which represents the 3'-half of a larger footprinted area (Box A, Fig. 7B). Gel mobility shift assays documented increased binding to the TGFβ-responsive element of nuclear proteins from TGFβ-treated cells compared with those from control cells (Fig. 7C). In the gel mobility shift assays, the slowest migrating band (top) corresponds to Sp1 bound to the 3A region, the middle band is C/EBP bound to the 5A region, and the fastest migrating band (bottom) are Smad3 and Smad4 bound to the B region (11, 12, 32, 38). The B region contains possible artificial binding sites for AP1 and NF-B when using short oligonucleotide probes. This same laboratory has demonstrated that C/EBPs are not essential for the TGFβ-mediated activation of the COL1A2 promoter, but they are for the TNFα repression of the COL1A2 promoter (11, 12, 32, 38). We therefore evaluated whether AdoMet modulates the levels of these proteins that bind to the TGFβ-responsive element of the COL1A2 promoter. Levels of Sp1, Smad3, and Smad4, and C/EBPβ and C/EBPδ were similar in HSC treated with TGFβ alone or cotreated with TGFβ plus AdoMet; therefore, AdoMet does not alter the availability of these transcription factors (Fig. 7D).

TGFβ induced the phosphorylation of p38 and ERK1/2 as early as 30 min. AdoMet prevented the phosphorylation of ERK1/2 but not that of p38 (Fig. 8A). Neither phosphatidylinositol 3-kinase nor phosphorylated Akt was affected by TGFβ or AdoMet treatment (data not shown). Addition of an inhibitor of ERK1/2 phosphorylation, PD98059, prevented the increase in collagen I by TGFβ (Fig. 8B), indicating that activated ERK1/2 is necessary for the induction of collagen I production by TGFβ. Phosphorylated ERK1/2 (pERK1/2) was immunoprecipitated and immunoblotted for either Sp1 or Smad3 and Smad4 to determine whether AdoMet prevention of phosphorylation of ERK1/2 could block its binding to either Sp1 or Smad3 or Smad4 transcription factors and the further transactivation of the COL1A2 promoter. pERK1/2 communoprecipitated with Sp1 (Fig. 8C) but not with Smad3 or Smad4 (not shown). This Sp1-pERK1/2 interaction was increased by TGFβ treatment of the HSC, and AdoMet completely blunted the TGFβ stimulation (Fig. 8C). Southwestern analysis demonstrated that pERK1/2 bound to Sp1 was able to bind the TGFβ-responsive element, and this binding was prevented by the addition of AdoMet (Fig. 8D).
DISCUSSION

During the course of liver fibrosis, AdoMet availability becomes compromised. AdoMet administration protects against liver damage elicited by a number of hepatotoxins, including ethanol, CCl4, galactosamine, acetaminophen, bile acids, or the administration of a choline-deficient diet (39–41). The beneficial effect of AdoMet against CCl4-induced liver fibrosis has been attributed to protecting AdoMet synthase from oxidation of a cysteine residue in the ATP binding site of the enzyme (42), to reconstitution of the reduced GSH pool (3, 7, 42, 43), lower lipid peroxidation as a result of AdoMet antioxidant actions (44), and to the capacity of preserving plasma membrane Na+/K+-ATPase. AdoMet administration also seems to restore levels of antioxidants such as vitamin E, which can inhibit stimulation of collagen synthesis in fibroblasts by decreasing lipid peroxidation (45), but other potential protective mechanisms are now emerging.

Several studies of transcriptional control of the murine COL1A2 suggest that there are two regions of promoter sequences, a proximal promoter between 378 and 58 bp, which is active in transfection assays and responsive to cytokines and ROS (46), and an upstream enhancer whose functional sequences are located between 17 and 15.5 kb (18). A critical element is located at 378 bp from the transcription start site because it contains a TNF, TGF, and ROS-sensitive region all of which can modulate collagen I levels (12, 29, 37). HSC are also activated by lipid peroxidation products such as malondialdehyde and 4-hydroxy-2,3-nonenal (47, 48), which also stimulate collagen I synthesis (49, 50). Up-regulation of TGFβ and COL1A1 and COL1A2 genes occurs in HSC during CCl4-induced fibrosis (51). Freshly isolated HSC produce little collagen I protein; but when cultured, they gradually produce collagen I while acquiring a phenotype resembling myofibroblasts. Such a phenotypic change of cultured HSC is thus considered a useful system to evaluate the mechanism underlying the process of hepatic fibrosis. With these two models in mind as possible working tools, we set out to analyze the hypothesis that AdoMet could prevent the fibrogenic response by lowering TGFβ levels and by repressing the COL1A2 promoter activation, and that a potential inhibition of the binding of TGFβ-sensitive transcription factors to the COL1A2 promoter may play a role in such regulation.
Fig. 6. Effect of AdoMet treatment on TGFβ levels and TGFβ-mediated induction of collagen I. A, mice treated with CCl₄ revealed intense positive TGFβ staining (dark color) around zones 1 and 3, whereas mice cotreated with CCl₄ plus AdoMet had reduced TGFβ staining (magnification, ×200) (n = 6). MO, mineral oil; SAM, S-adenosylmethionine. B, similarly, AdoMet lowered the CCl₄-induced elevation of TGFβ protein as detected by Western blot analysis (n = 1). C, HSC were incubated in the absence or presence of AdoMet (30 μM, daily addition), and samples of culture medium were evaluated for TGFβ content by enzyme-linked immunosorbent assay. Results are expressed as pg/mL. Solid line, control HSC; broken line, HSC + AdoMet. D, primary HSC were incubated in the presence or absence of 30 μM AdoMet 24 h prior to the addition of TGFβ (10 ng/ml) followed by an additional 24-h incubation, and collagen I levels were analyzed by Western blot. Numbers under the blots refer to AU of densitometry for each protein normalized to β-tubulin. ●●, p < 0.01 for AdoMet versus not treated HSC; *** , p < 0.001 for TGFβ versus not treated HSC; and ▲▲, p < 0.01 for AdoMet + TGFβ versus TGFβ.
As a first approach, transgenic mice harboring the full-length mouse COL1A2 promoter upstream sequence linked to the /H9252-gal reporter gene were used. Liver fibrosis was induced by injection of CCl4. Injury was observed by increased levels of transaminases, H&E staining, endogenous total collagen deposition, and elevated TGF/H9252 expression as has also been observed by others (52, 53). Coadministration of AdoMet ameliorated these effects. Induction of the COL1A2 promoter was observed in HSC from the CCl4-treated transgenic mice, and AdoMet blocked such activation as demonstrated by /H9252-gal staining, activity, and colocalization with /H9251-Sma, a marker for activation of HSC.

Several potential mechanisms are likely to contribute to the protective actions of AdoMet. Although the CCl4-induced decrease in hepatic GSH and increase in lipid peroxidation were modest, AdoMet reduced lipid peroxidation and increased GSH.
content to above control values; these most likely reflect antioxidant actions of AdoMet and replenishment of GSH. Even though AdoMet alone had no effect, AdoMet plus CCl₄ downregulated the activity and protein expression of CYP2E1 over the decrease produced by CCl₄ alone. This further lowering of CYP2E1, a potential source of oxidative stress, may participate in the protective actions of AdoMet because of the sensitivity of the COL1A2 promoter to ROS and to ROS-activated cytokines (e.g. TNFα and TGFβ) (11, 12, 21, 32, 47, 49). Importantly, AdoMet lowered the CCl₄-induced elevation of TGFβ to control levels. Thus, it is likely that several factors combine to promote the protective actions of AdoMet in vivo.

As a second approach and to gain mechanistic insight, HSC cultured in the presence or absence of AdoMet were studied.

There was a time- and dose-dependent down-regulation of intracellular and secreted collagen I and α-Sma proteins in the presence of AdoMet. Furthermore, two metabolites of AdoMet, SAH and MTA, were able to replicate these same effects at the protein and mRNA level. Methionine, an AdoMet precursor, also lowered collagen I protein and mRNA although less effectively, probably because of the minimal methionine adenosyltransferase 1A activity in HSC (36). Because SAH and MTA, unlike AdoMet, are not methylating agents, unless they generate AdoMet again, it is likely that the down-regulation of collagen I protein and mRNA by AdoMet may not be the result of gene silencing via methylation.

To assess whether the down-regulation of collagen I could be caused, at least in part, by repression of the collagen I promoter

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**FIG. 8.** AdoMet blocks ERK1/2 phosphorylation in TGFβ-treated HSC and lowers Sp1 binding to the COLIA2 promoter. A, primary HSC were isolated, placed in culture for 3 days, and treated with 30 μM AdoMet 24 h prior to the addition of 10 ng/ml TGFβ. Samples were collected at 30, 60, and 120 min or 24 h. The figure depicts a Western blot analysis for collagen I, phosphorylated and total ERK1/2, and p38 under TGFβ and TGFβ plus AdoMet treatment. Numbers under the blots refer to AU of densitometry for collagen I and the p38 to total p38 ratio or the pERK1/2 to total ERK1/2 ratio. SAM, S-adenosylmethionine. B, PD98059, an inhibitor of ERK1/2 phosphorylation, prevents the TGFβ-mediated increase in collagen I protein (n = 1). C, pERK1/2 forms a complex with Sp1. pERK1/2 was immunoprecipitated and immunoblotted for Sp1 (n = 1). D, Southwestern analysis of the pERK1/2-Sp1 complex bound to the probe containing the TGFβ-responsive element (RE) from the COLIA2 promoter. In all cases numbers under the blots refer to AU of densitometry with the control not treated HSC assigned a value of 1 (n = 1).
(COL1A1 and COL1A2 mRNA levels were decreased by AdoMet), HSC were transfected with a series of deletion constructs for the human COL1A2 promoter. There was repression of the basal promoter activity in the presence of AdoMet. The −378 to +58 bp of the human COL1A2 promoter contains a TGFβ (−313 to −250), TNFα, and a ROS-responsive element, and it is critical for basal and induced responsiveness (29, 54, 55). The in vitro experiments were not carried out under CCl4 treatment because of the lack of cytochrome P450s, which carry out CCl4 metabolism in HSC.

Based on the low TGFβ expression in livers of mice injected with CCl4 and cotreated with AdoMet, we measured the concentration of TGFβ secreted to the culture medium by HSC treated with AdoMet and found only a slight decrease compared with control values. Therefore, it is unlikely that AdoMet inhibition of TGFβ production by HSC plays a major role in the down-regulation of collagen I production. The possibility that AdoMet could prevent the mechanisms and signaling pathways by which TGFβ increases collagen I protein levels as a protective mechanism was then evaluated. When HSC were pre-incubated with AdoMet 24 h prior to the addition of TGFβ, collagen I expression was lowered to levels found in control HSC not treated with TGFβ, indicating that AdoMet prevents the stimulatory actions of TGFβ on collagen I production.

A reporter assay using the −378 to +38 COL1A2-CAT promoter region transfected into HSC and then treated with AdoMet plus TGFβ showed repression of the basal and TGFβ-stimulated CAT activity. Furthermore, binding assays using the TGFβ-responsive element as a probe indicated that administration of AdoMet could prevent TGFβ-sensitive proteins from binding to this site in the human COL1A2 promoter as confirmed by competition studies with cold probe containing the TGFβ-responsive element. Sp1, C/EBPβ, and C/EBPδ, and Smad3 and Smad4 availability was similar under TGFβ pretreatment of TGFβ/H9252 and its metabolites on TGFβ/H9252 stimulation of the AdoMet and its metabolites on TGFβ/H9252.

The protective mechanism of AdoMet against liver fibrosis may be to reduce TGFβ-stimulated CAT activity. Whether AdoMet further modifies the recruitment, affinity, and binding of other cofactors repressing the COL1A2 gene transcription is also conceivable. In addition, although these results demonstrated that AdoMet protected from a fibrogenic stimulus via decreasing TGFβ levels and the interaction of TGFβ with the COL1A2 promoter, the possibility of post-translational modifications on collagen I protein, secretion, and extracellular degradation affecting the net accumulation of collagen I in the space of Disse is not ruled out.

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