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Effect of *S*-adenosyl-L-methionine on the activation, proliferation and contraction of hepatic stellate cells

Hiroko Matsui[a,b], Norifumi Kawada[c,*]

[a]*Department of Anatomy, Graduate School of Medicine, Osaka City University, Osaka, Japan*
[b]*Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan*
[c]*Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3, Asahimachi, Abeno, Osaka 545-8585, Japan*

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Abstract

Inhibition of hepatic stellate cell activation is an important clinical aspect for the control of liver inflammation, fibrosis and cirrhosis. *S*-adenosyl-L-methionine (SAM), an intermediate product of L-methionine metabolism, is a precursor of glutathione and an endogenous methyl donor. Although the hepato-protective action of SAM has been reported in several animal models, the effect of SAM on the function of hepatic stellate cells has not been elucidated. Using a primary-culture model of hepatic stellate cells, we found that SAM blunts the activation process as indicated by the suppression of expression of collagen α1(I) and smooth muscle α-actin. SAM also hampers the DNA synthesis of hepatic stellate cells stimulated with a dimer of platelet-derived growth factor-B via the inhibition of phosphorylation of PDGF receptor-β and down-stream signaling pathways. SAM additionally inhibits the contraction of hepatic stellate cells by disturbing the formation of F-actin stress fibers and phosphorylated myosin light chains. Thus, SAM regulates the activation of hepatic stellate cells and may clinically contribute to therapy targeted at human liver fibrosis.

Keywords: Dimer of platelet-derived growth factor-B; Collagen; Smooth muscle α-actin; Endothelin-1; L-Methionine; L-Cysteine

1. Introduction

Hepatic stellate cells reside in sinusoids, maintain a quiescent phenotype, and store vitamin A (Okuyama et al., 2002; Eng and Friedman, 2000). In response to inflammatory stimuli, hepatic stellate cells undergo activation and proliferate, contract, secrete profibrogenic mediators, and generate extracellular matrix materials as well as play a role in the fibrosis of the liver (Friedman, 2000; Gabele et al., 2003; Pinzani and Marra, 2001). The suppression of hepatic stellate cell activation is thus a clinical issue which requires the establishment of therapeutic strategies against liver fibrosis (Albanis and Friedman, 2001; Bataller and Brenner, 2001).

Our previous studies have shown that L-cysteine, L-methionine and *N*-acetyl-L-cysteine exert anti-fibrotic activity in the liver (Kawada et al., 1998; Okuyama et al., 2001; Matsui et al., 2004). Oral supplementation of these amino acids suppressed septum formation and hepatic hydroxyproline content as well as mRNA expression of type I collagen, transforming growth factor beta (TGFβ) and tissue inhibitor of matrix metalloproteinases (TIMPs) in a thioacetamide-induced model of liver cirrhosis in rats. Mechanical analyses have confirmed that these amino acids directly affect the activation of primary-cultured hepatic stellate cells. Similar results were reported for different experimental conditions (Kim et al., 2001).

L-Methionine is metabolized to *S*-adenosyl-L-methionine (SAM) via methionine adenosyltransferase in the liver and in various extrahepatic organs (Avila et al., 2002; Lieber, 2002). In addition to its activity as a methyl donor, SAM has anti-inflammatory properties in the liver and is a precursor of glutathione. Thus it protects hepatocytes from oxidative stress (Lu et al., 2000, 2001; Martinez-Chantar et al., 2002;

* Corresponding author. Tel.: +81 6 6645 3811; fax: +81 6 6645 3813.
E-mail address: kawadanori@med.osaka-cu.ac.jp (N. Kawada).

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Halsted et al., 2002; Mato et al., 1999; Gasso et al., 1996; Varela-Moreiras et al., 1995). However, its action on constituent hepatic cells except for hepatocytes is largely unknown. Here, we report the inhibitory effect of SAM on the activation-associated function of primary cultured hepatic stellate cells.

2. Materials and methods

2.1. Materials

Collagenase and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) were purchased from Wako Pure Chemical (Osaka, Japan). Pronase E was aquired from Merck (Damstadt, Germany), and recombinant rat dimer of platelet-derived growth factor-B (PDGF-BB) was obtained from R&D Systems (Minneapolis, MO). Polyclonal antibodies against extracellular signal-regulated kinases 1 and 2 (ERK1/2), phospho-ERK1/2 (Thr 202/Tyr 204), Akt and phospho-Akt (Ser 473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and those against PDGF receptor-β, TGF-β receptor type II (TGFβRII), myosine light chain and phospho-myosine light chain were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against smooth muscle α-actin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, TRITC-labeled phalloidin and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma (Saint Louis, MO), and rat endothelin-1 was obtained from Peptide Institute (Osaka, Japan). Antibodies against STAP (stellate cell activation-associated protein) were generated in our laboratory as previously described (Kawada et al., 2001). Enhanced chemiluminescence detection reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). A GeneAmp RNA polymerase chain reaction (PCR) Core Kit was obtained from PerkinElmer Life Sciences (Boston, MA), and isogen and agarose S were acquired from Nippon Gene (Tokyo, Japan). Immobilon P membranes were purchased from Millipore (Bedford, MA). Kodak XAR5 film was purchased from Eastman Kodak (Rochester, NY). All the other reagents were obtained from Sigma or Wako Pure Chemical.

2.2. Animals

Pathogen-free male Wistar rats were obtained from SLC (Shizuoka, Japan). The experimental protocols were approved by the Animal Research Committee of Osaka City University (Guide for Animal Experiments, Osaka City University).

2.3. Preparation of hepatic stellate cells

Hepatic stellate cells were isolated from male Wistar rats as previously described (Kristensen et al., 2000) and then cultured on plastic dishes in DMEM supplemented with 10% fetal bovine serum. After culturing had continued for the indicated number of days, the medium was replaced by serum-free DMEM with test agents and the culture was continued for 48 h.

2.4. BrdU incorporation assay

The incorporation of BrdU was immunocytochemically evaluated as previously described (Uyama et al., 2002). BrdU-positive cells were counted under four randomly chosen microscopic fields in each well. A BrdU labeling index (BrdU L.I.) was calculated as the number of BrdU-positive cells/number of cells in one area ×100 (%).

2.5. Immunoblot

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Immobilon P membranes. After blocking, the membranes were treated with primary antibodies followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and Kodak XAR5 film.

2.6. RT-PCR

mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) using a GeneAmp RNA PCR Core Kit as previously described (Uyama et al., 2002). PCR products were then separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The primers used are listed in Table 1.

2.7. F-actin staining

F-actin in formalin-fixed cells was stained using TRITC-phalloidin as previously described and observed under a fluorescent microscope (Kawada et al., 1996a).

Table 1
Primer pairs used for RT-PCR in this study

	Forward	Reverse	Access no.
Smooth muscle α-actin	5′-TGTGCTGGACTCTGGAGATG-3′	5′-GATCACCTGCCCATCAGG-3′	X06801
Collagen α1(I)	5′-TGCCGTGACCTCAAGATGTG-3′	5′-CACAAGCGTGCTGTAGGTGA-3′	M11432
GAPDH	5′-GATGCTGGTGCTGAGTATGT-3′	5′-TCATTGAGAGCAATGCCAGC-3′	X02231

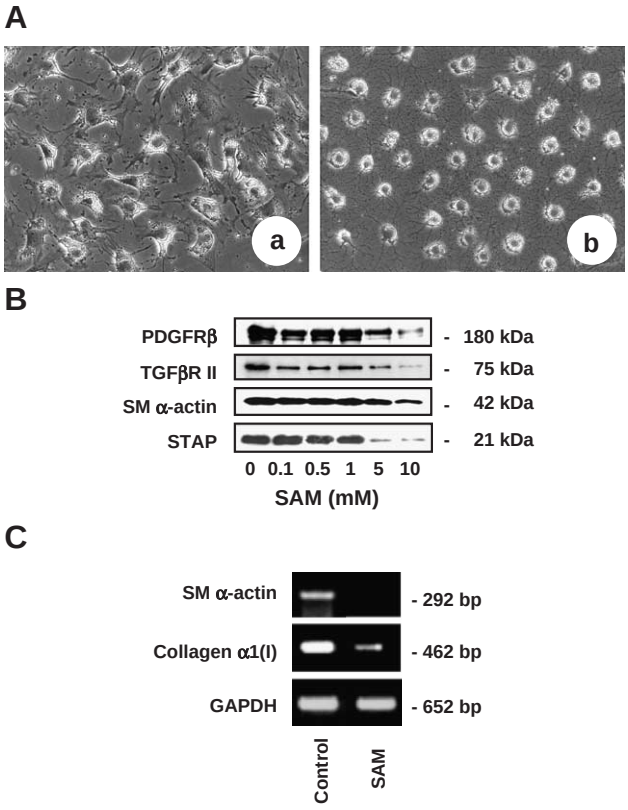

Fig. 1. The effect of SAM on the morphology of primary-cultured hepatic stellate cells and their expression of activation markers. (A) Hepatic stellate cells were freshly isolated from intact rat liver and allowed to adhere to culture plates in the presence of 10% fetal bovine serum/DMEM for 24 h. Then, the culture medium was replaced with serum-free DMEM supplemented with SAM, and was subsequently changed everyday. Culturing was continued for 4 days. The morphology of the hepatic stellate cells was observed by phase-contrast microscopy and photographed. a: Untreated control culture on day 4. b: Addition of 5 mM SAM. (B) Expression of PDGF receptor β, TGFβ receptor II, smooth muscle α-actin and STAP. Hepatic stellate cells were isolated, cultured and treated as in (A) and cell lysates were prepared on day 4. Protein expression was analyzed by Western blotting. Experiments were repeated 3 times. Representative data are presented here. SM α-actin, smooth muscle α-actin. (C) The effect of SAM on the expression of smooth muscle α-actin and collagen α1(I) mRNA. Hepatic stellate cells were cultured in serum-free DMEM supplemented with 10 mM SAM as in (A) and total RNA was prepared on day 4. Expression of smooth muscle α-actin and collagen α1(I) mRNA was analyzed by RT-PCR. Experiments were repeated 3 times. Representative data are presented here. GAPDH, glyceroaldehyde-3-phosphate dehydrogenase, was used as a house-keeping gene.

2.8. Detection of cell contraction

The contraction of hepatic stellate cells was detected using a hydrated collagen lattice method as previously described (Kawada et al., 1995, 1996b). The diameter of the collagen gel was measured 24 h after the addition of 1 nM endothelin-1.

2.9. Statistical analysis

Data presented as bar graphs are the means±S.D. of at least three independent experiments. Statistical analysis was performed with Student's *t*-test ($P<0.01$ was considered significant).

3. Results

3.1. Effect of SAM on the activation of hepatic stellate cells

As shown in Fig. 1Aa, hepatic stellate cells in primary-culture for 4 days after plating exhibited a myofibroblast-like configuration with enlarged nuclei and expanded processes, but still stored cytoplasmic lipid droplets that contain vitamin A. Hepatic stellate cells exposed to SAM maintained a quiescent morphology with small nuclei and branching processes, very similar to the shape of fresh hepatic stellate cells after isolation (Fig. 1Ab). Trypan blue assay revealed that there was no significant difference in cell viability between control and SAM-treated hepatic stellate cells (data not shown). Western blot analysis revealed that the expression of PDGF receptor β, TGFβ receptor II,

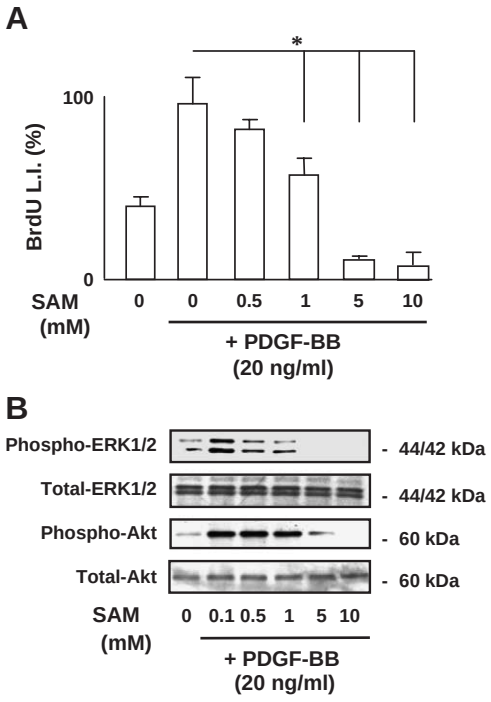

Fig. 2. The effects of SAM on hepatic stellate cell proliferation under PDGF-BB stimulation. (A) BrdU incorporation assay. Hepatic stellate cells at 7.5×10^5 cells/well were incubated in serum-free DMEM supplemented with the indicated doses of SAM for 24 h and then cultured in the same medium containing 100 μM BrdU in the presence of 20 ng/ml of PDGF-BB for 24 h. Incorporated BrdU was immunocytochemically evaluated and the BrdU labeling index (BrdU L.I.) was calculated. Data from 5 individual experiments are shown. *$P<0.01$. (B) The effect of SAM on the phosphorylation of ERK1/2 and Akt on stimulation with 20 ng/ml of PDGF-BB. Hepatic stellate cells were treated with SAM at the indicated doses for 48 h in serum-free DMEM and successively stimulated with PDGF-BB for 10 min. Phospho- and total-proteins were analyzed by Western blotting. Experiments were repeated 3 times. Representative data are presented here.

smooth muscle α-actin and STAP, which is known to be induced during the activation of hepatic stellate cells, was reduced when the cells were incubated in the presence of SAM in a dose-dependent manner. In addition, SAM apparently reduced the expression of smooth muscle α-actin and collagen α1(I) mRNA in hepatic stellate cells as revealed by the RT-PCR analysis (Fig. 1C).

3.2. Effect of SAM on the PDGF-BB-stimulated proliferation of activated hepatic stellate cells

The proliferation of hepatic stellate cells in response to PDGF-BB is a feature of cell activation. As shown in Fig. 2A, SAM inhibited DNA synthesis in activated hepatic stellate cells stimulated with 20 ng/ml of PDGF-BB in a dose-dependent manner. Mechanical analyses revealed that the phosphorylation of ERK1/2 and Akt on stimulation with PDGF-BB was attenuated in the presence of SAM without affecting the total amount of protein produced (Fig. 2B).

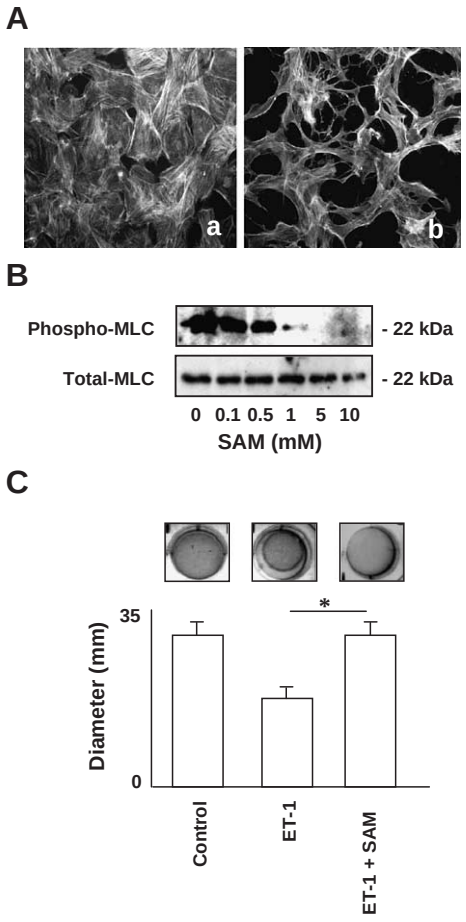

Fig. 3. The effects of SAM on the contraction of activated hepatic stellate cells. (A) Activated hepatic stellate cells were cultured in serum-free DMEM supplemented with 5 mM SAM for 48 h. The cells were then fixed with 4% formalin and after being washed with PBS were stained with 0.5 μg/ml of TRITC-phalloidin. After another wash with PBS, they were observed by fluorescent microscope. (B) Activated hepatic stellate cells were treated with SAM at the indicated doses for 48 h in serum-free DMEM and phospho- and total-MLC were then analyzed by Western blotting. Experiments were repeated 3 times. Representative data are presented here. MLC, myosin light chain. (C) Activated hepatic stellate cells (1×10^6 cells) were introduced onto the surface of hydrated collagen lattices. The cells were then treated with 1 nM endothelin-1 and 5 mM SAM in serum-free DMEM for 48 h. The diameter of the gels (original diameter=35 mm) was measured using photographs. Experiments were repeated 3 times. Representative data are presented here. ET-1, endothelin-1.

3.3. Effect of SAM on the endothelin-1-induced contraction of activated hepatic stellate cells

The contraction of hepatic stellate cells is an additional property of the activated phenotype and plays a role in the regulation of sinusoidal microcirculation, and portal hypertension in the cirrhotic liver. Thus, we tested whether SAM can modify the contractile activity of activated hepatic stellate cells. As shown in Fig. 3Aa, activated hepatic stellate cells exhibited well-organized bundles of F-actin stress fibers. SAM treatment led to a transformation of cell shape accompanied by the disruption of these fibers (Fig. 3Ab). This occurred in part due to the de-phosphorylation of the myosine light chain as shown in Fig. 3B. In accordance with this, SAM significantly suppressed the contractile potency of hepatic stellate cells stimulated by endothelin-1 (Fig. 3C).

4. Discussion

In this study, we showed that SAM, an intermediate product in L-methionine metabolism, attenuates hepatic stellate cell activation, the response to PDGF-BB and subsequent proliferation and contractile activity. There are a few reports on the effects of amino acids and their metabolites on hepatic stellate cell function and liver pathophysiology. L-Leucine stimulates the secretion of hepatocyte growth factor by hepatic stellate cells (Tomiya et al., 2002), whereas *N*-Acetyl-L-cysteine and L-cysteine suppress the proliferation of primary-cultured hepatic stellate cells and inhibit liver fibrosis in rat models (Kawada et al., 1998; Okuyama et al., 2001; Matsui et al., 2004; Kim et al., 2001). Thus, the data presented here on SAM provides new insight into the pharmacological action of amino acids and their intermediate metabolites in liver disease and on hepatic stellate cell function.

Although we used rather high concentrations of SAM in the present study, we observed that SAM had no toxic effect on cultured hepatic stellate cells, as revealed by Trypan blue staining. In our previous study, we also used a few mM of L-methionine and L-cycteine without any toxic effect (Matsui et al., 2004). Such high concentrations of SAM have been used in several in vitro studies (Majano et al., 2001; Yang et al., 2004).

L-Methionine is a component of proteins and is used as a methyl donor after its conversion to SAM via methionine adenosyltransferase (Avila et al., 2002; Lieber, 2002; Lu et al., 2000, 2001; Martinez-Chantar et al., 2002; Halsted et

al., 2002; Mato et al., 1999; Gasso et al., 1996; Varela-Moreiras et al., 1995). While SAM prevents hepatic fibrosis induced in the rat by CCl_4 intoxication (Gasso et al., 1996; Varela-Moreiras et al., 1995), direct evidence of SAM activity in the activation of hepatic stellate cells has not been reported until now. However, as recently reported, spontaneous steatohepatitis accompanied with fibrosis occurs in methionine adenosyltransferase 1 knockout mice (Santamaria et al., 2003), indicating that SAM plays a protective role in inflammatory reactions in the liver. Since the effect of SAM on hepatic stellate cell function was similar to that of L-cysteine as reported (Matsui et al., 2004), we speculate that SAM is metabolized to L-cysteine via the transsulfuration pathway in hepatic stellate cells and acts as a reducing agent that regulates the cellular response to growth factors. In this context, although we did not measure the intracellular glutathione content in SAM-treated hepatic stellate cells, SAM would be converted to glutathione, one of the most important reducing molecules. However, the action of SAM as a methyl donor should be taken into account because methylation of CpG islands in DNA can contribute to the regulation of transcriptional bursts in the early stages of hepatic stellate cell activation (Chen et al., 2004).

In summary, we showed here the inhibitory effect of SAM on the activation process in cultured hepatic stellate cells. Since SAM is used in clinical practice in Europe for liver disease, this agent will be of benefit in protecting the liver against fibrosis.

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