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Pepstatin A attenuates the inhibitory effect of *N*-acetyl-L-cysteine on proliferation of hepatic myofibroblasts (stellate cells)

Tokuko Takashima [a], Norifumi Kawada [a,*], Naoto Maeda [a], Hiroaki Okuyama [b], Naoki Uyama [b], Shuichi Seki [a], Tetsuo Arakawa [a,c]

[a] *Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno 545-8585 Osaka, Japan*
[b] *Department of Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan*
[c] *Department of Gastroenterology, Graduate School of Medicine, Osaka City University, Osaka, Japan*

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Abstract

The pharmacological interaction between pepstatin A, a specific inhibitor of cathepsin D, and *N*-acetyl-L-cysteine was analyzed using hepatic stellate cells in primary culture. Isolated rat stellate cells were cultured on plastic dishes in Dulbecco's modified Eagle's medium (DMEM). Proteins and phospho-proteins were detected by Western blot. DNA synthesis was determined by [^{3}H]thymidine uptake. Pepstatin A restored DNA synthesis of stellate cells stimulated by either platelet-derived growth factor-BB (PDGF-BB) or insulin-like growth factor-I (IGF-I), an effect that was attenuated by *N*-acetyl-L-cysteine. This agent induced the recovery of both the expression of PDGF receptor β and IGF-I receptor β and the phosphorylation of p42/44 mitogen-activated protein kinase (MAPK) and Akt under stimulation with either PDGF-BB or IGF-I, which were downregulated by *N*-acetyl-L-cysteine. Expression of cathepsin D was induced in activated stellate cells. These results indicate that pepstatin A hampers the inhibitory effect of *N*-acetyl-L-cysteine on stellate cell growth by ameliorating growth factor receptor downregulation.

Keywords: Pepstatin A; *N*-acetyl-L-cysteine; Stellate cell; Cathepsin D; IGF-I (insulin-like growth factor-I); PDGF (platelet-derived growth factor)

1. Introduction

Analysis of the molecular mechanism of the activation of stellate cells, liver-specific myofibroblasts, has been urged in order to establish a therapeutic strategy for hepatic fibrosis. Cell activation coincides with loss of retinoids, enhanced production of extracellular matrix materials including fibril-forming collagens, proliferation, cellular contractility associated with smooth muscle α-actin expression, generation of inflammatory mediators such as chemokines and growth factors, and production of extracellular matrix material degradation-related molecules such as matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Friedman, 1993, 2000; Kawada, 1997; Pinzani and Marra, 2001; Rockey, 2001).

Platelet-derived growth factor (PDGF) and insulin-like growth factor-I (IGF-I) stimulate the proliferation of stellate cells in a paracrine and an autocrine manner (Bissell et al., 1995; Okuno et al., 1997; Pinzani et al., 1989, 1996; Skrtic et al., 1997; Svegliati-Baroni et al., 1999). Expression of the individual receptors, such as PDGF receptor β and IGF-I receptor β, and signaling pathways after ligand–receptor coupling, such as Raf-1/mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase (MAPK) cascade and phosphatidylinositol 3-kinase (PI3-K)/Akt cascade, have been intensively analyzed in stellate cells. On the basis of these findings, in order to inhibit stellate cell proliferation, thereby hampering the progress of liver fibrosis, antigrowth factor strategies have been proposed and are under clinical investigation.

N-acetyl-L-cysteine is widely used as a glutathione precursor and works as a reducing agent. This agent scavenges hydrogen peroxide and therefore is called an antioxidant. It is also clinically used, for instance, against acetaminophen-induced acute liver injury. We and others have previously

* 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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demonstrated that *N*-acetyl-L-cysteine effectively suppresses stellate cell proliferation and activation-dependent expression of smooth muscle α-actin in these cells, indicating that *N*-acetyl-L-cysteine could be a candidate for the clinical treatment of liver fibrosis (Kawada et al., 1998; Kim et al., 2001). Although the latter report showed that these effects of *N*-acetyl-L-cysteine were caused by its radical scavenging action, the pharmacological action of *N*-acetyl-L-cysteine on stellate cells remains to be characterized further. Here, we show that *N*-acetyl-L-cysteine downregulated the expression of growth factor receptors in stellate cells, a response that was hampered by pepstatin A, a selective inhibitor of cathepsin D.

2. Material and methods

2.1. Animals

Male Wistar rats (about 300–340 g body weight) were obtained from SLC (Shizuoka, Japan). Animals were housed at a constant temperature and supplied with laboratory chow and water ad libitum. The protocol of experiments was approved by The Animal Research Committee, Guide for Animal Experiments, Osaka City University.

2.2. Chemicals

Pronase E was purchased from Merck (Darmstadt, FRG). DNase was from Boehringer Mannheim (Mannheim, FRG). Collagenase, isogen and thioacetamide were from Wako (Osaka, Japan). Nycodenz was from Nycomed Pharmas (Oslo, Norway). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco BRL (Gaithersburg, MD). [*Methyl*-^{3}H]thymidine (40–60 Ci/mmol) and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Monoclonal antibodies against cathepsin D, PDGF receptor β, and IGF-I receptor β were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against smooth muscle α-actin was from DAKO (Glostrup, Denmark). Polyclonal antibodies against Akt, phospho-Akt, p42/44 MAPK, and phospho-p42/44 MAPK were from New England Biolabs (Beverly, MA). Pepstatin A was from Peptide Institute (Osaka, Japan).

2.3. Isolation and culture of stellate cells

Stellate cells were obtained from normal rat livers as previously described (Kawada et al., 1993). Briefly, normal rat livers were perfused with Ca^{2+}/Mg^{2+}-free Gey's balanced salt solution, followed by digestion with 0.1% pronase and 0.04% collagenase for 40 min at 37 °C. The digested liver was excised, cut into small pieces, and incubated in Gey's balanced salt solution containing 0.08% pronase E, 0.08% collagenase, and 20 μg/ml of DNase. The resulting suspension was filtered through a 150-μm nylon mesh and centrifuged on an 8.2% Nycodenz cushion, which produced a stellate cell-enriched fraction in the upper-whitish layer. The cells were washed, suspended in DMEM supplemented with 10% fetal bovine serum, 10^5 U/l penicillin G, and 100 mg/l streptomycin, and plated on plastic culture dishes (Falcon 3003, Beckton Dickinson, Flanklin Lakes, NJ). Cell purity was always more than 95%, as assessed by a typical starlike configuration and by detecting vitamin A autofluorescence. The yield of stellate cells was always about 20 million cells from a normal liver. After plating, the stellate cells were allowed to attach to the culture dishes for 24 h in order to remove dead cells and debris. The culture medium in the presence or absence of test agents was changed every other day, unless otherwise mentioned. Stellate cells cultured for 2 days were referred to as quiescent stellate cells, and cells cultured for 7–8 days were referred to as activated ones. Quiescent stellate cells possessed lipid particles containing vitamin A and extended dendritic processes from the cell bodies. Activated ones lost the particles and looked very similar to fibroblasts. These activated stellate cells, but not quiescent ones, were positive for smooth muscle α-actin (Kawada, 1997).

2.4. Generation of liver fibrosis

A liver fibrosis model was prepared in rats by administering 50 mg/body of thioacetamide two times a week for 8 weeks. The development of fibrosis was estimated histologically using Azan Mallory staining.

2.5. Western blot analysis

Stellate cells were cultured for the indicated periods in the presence or absence of test agents, and then homogenized in buffer [62.5 mM Tris, 0.1% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-β mercaptoethanol, pH 6.8]. After heat-denaturation (95 °C for 5 min), the samples (10 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto an Immobilon P membrane (Millipore, Bedford, MA). After washing and blocking, the membranes were treated with monoclonal antibodies against smooth muscle α-actin, cathepsin D, PDGF receptor β, IGF-I receptor β, total-Akt, phospho-Akt, total-MAPK, or phospho-MAPK overnight at 4 °C. After washing, individual membranes were reacted with horseradish peroxidase-labeled secondary antibodies for 2 h at room temperature. Immunoreactive bands were visualized on Kodak XAR5 films using ECL detection reagent (Amersham Pharmacia Biotech). The developed films were subjected to densitometric analysis.

2.6. [^{3}H]thymidine incorporation assay

Stellate cells cultured in 35-mm plates were incubated with 1.0 μCi/ml of [^{3}H]thymidine. After cells were washed

with phosphate-buffered saline (PBS) three times, reactions were terminated by adding 1 ml of 10% trichloroacetic acid. The plates were incubated for 2 h at 4 °C. After aspiration of trichloroacetic acid, each well was washed with PBS three times and the resultant acid-insoluble materials were lyzed with 0.5 ml of 0.5 N NaOH. The solubilized materials were mixed with 5 ml of Aquasol-2 (DuPont) after adjustment of the pH to 7.0. The radioactivity of each sample was counted in an LS 7500 liquid-scintillation analyzer (Beckman).

2.7. Statistical analysis

Values are expressed as means ± S.E. Statistical analysis was performed by Fisher's PLSD (protected least significant difference). *P*-values less than 0.05 were considered significant.

3. Results

3.1. Effect of pepstatin A on DNA synthesis of stellate cells

As shown in Fig. 1, *N*-acetyl-L-cysteine attenuated the DNA synthesis of both nonstimulated and growth factor-stimulated rat stellate cells in primary culture. In the course of the analysis of this *N*-acetyl-L-cysteine-dependent inhibition of DNA synthesis, we found that pepstatin A, a specific inhibitor of cathepsin D, hampered the action of *N*-acetyl-L-cysteine. Although pepstatin A alone had a negligible effect on the DNA synthesis of stellate cells, it significantly ameliorated the *N*-acetyl-L-cysteine-induced attenuation of DNA synthesis under stimulation with either IGF-I (100 ng/ml) or PDGF-BB (20 ng/ml). For instance, *N*-acetyl-L-cysteine reduced DNA synthesis of stellate cells from a control level of 100 ± 6.44% to 38.9 ± 6.44% even under IGF-I stimulation. However, addition of pepstatin A improved the level to 139 ± 13.4%. Similarly, pepstatin A restored the DNA synthesis of stellate cells stimulated by

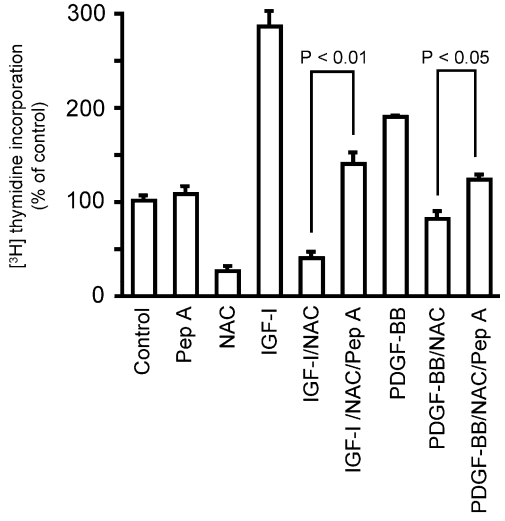

Fig. 1. Effect of pepstatin A on the DNA synthesis of stellate cells. Stellate cells treated with either *N*-acetyl-L-cysteine (20 mM) or *N*-acetyl-L-cysteine (20 mM)/pepstatin A (20 μM) for 24 h were successively stimulated with either IGF-I (100 ng/ml) or PDGF-BB (20 ng/ml) for the following 48 h. The cells were labeled with [^{3}H]thymidine (1 μCi/ml) for the last 24 h. Radioactivity in the cell lysate was counted using a liquid-scintillation analyzer. Note that pepstatin A significantly restored the DNA synthesis of stellate cells that was downregulated by *N*-acetyl-L-cysteine treatment. NAC, *N*-acetyl-L-cysteine. Pep A, pepstatin A. Means ± S.E.M. of five individual experiments are indicated.

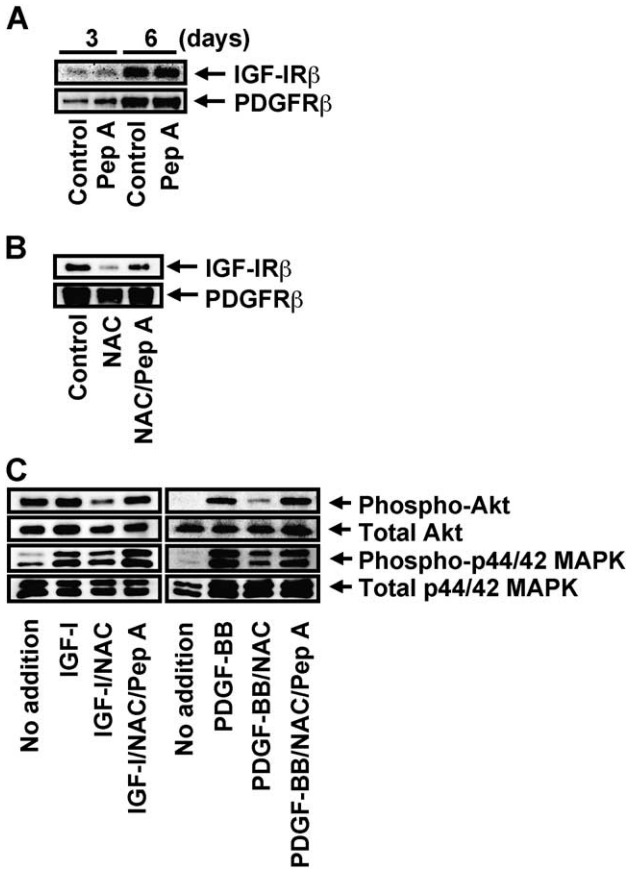

Fig. 2. Effect of pepstatin A on the expression of PDGF receptor β and IGF-I receptor β and on the phosphorylation of MAPK and Akt. (A) Stellate cells cultured for 3 or 6 days were treated with 20 μM pepstatin A, a selective inhibitor of cathepsin D, for 24 h. Cells were lyzed and the expression of PDGF receptor β and IGF-I receptor β was determined by Western blot. PDGFRβ, PDGF receptor β. IGF-IRβ, IGF-I receptor β. Pep A, pepstatin A. (B) Effect of pepstatin A on the expression of PDGF receptor β and IGF-I receptor β that was downregulated by the treatment with *N*-acetyl-L-cysteine (10 mM) for 24 h. Note that the expression of both PDGF receptor β and IGF-I receptor β was downregulated by *N*-acetyl-L-cysteine, while addition of pepstatin A restored protein expression. PDGFRβ, PDGF receptor β. IGF-IRβ, IGF-I receptor β. NAC, *N*-acetyl-L-cysteine. Pep A, pepstatin A. (C) Effect of pepstatin A on the phosphorylation of p42/44 MAPK and Akt under stimulation with IGF-I and PDGF-BB. Either IGF-I (100 ng/ml) or PDGF-BB (10 ng/ml) stimulated the occurrence of phopho-p42/44 MAPK and phopho-Akt in stellate cells at 10 min after exposure. The activation of MAPK and Akt was significantly hampered under the reducing conditions generated in the presence of *N*-acetyl-L-cysteine (10 mM). Pepstatin A (20 μM) restored the expression of phospho-p42/44 MAPK and phopho-Akt even in the presence of *N*-acetyl-L-cysteine. NAC, *N*-acetyl-L-cysteine. Pep A, pepstatin A.

PDGF-BB in the presence of *N*-acetyl-L-cysteine from 79.8 ± 10.2% to 122 ± 6.03%.

3.2. Effect of pepstatin A on the expression of PDGF receptor β and IGF-I receptor β in stellate cells

The results shown above indicated that *N*-acetyl-L-cysteine modulated the response of stellate cells to PDGF-BB and IGF-I, an effect that was reversed by pepstatin A. To investigate further the mechanism, we then checked the change in the level of growth factor receptors in stellate cells treated with *N*-acetyl-L-cysteine and pepstatin A. Although pepstatin A (20 μM) had no effect on the expression of PDGF receptor β and IGF-I receptor β (Fig. 2A), pepstatin A was found to restore the levels of both receptors after these had been suppressed by *N*-acetyl-L-cysteine, as revealed by Western blot (Fig. 2B).

3.3. Effect of pepstatin A on signal transduction under IGF-I and PDGF-BB stimulation

As shown in Fig. 2C, both p42/44 MAPK and Akt were phosphorylated under stimulation with either PDGF-BB (20 ng/ml) or IGF-I (100 ng/ml) in stellate cells. Treatment of the cells with *N*-acetyl-L-cysteine attenuated the expression of phospho-p42/44 MAPK and phospho-Akt. However, addition of pepstatin A (20 μM) to the culture medium markedly restored the expression of both phospho-p42/44 MAPK and phospho-Akt (Fig. 2C).

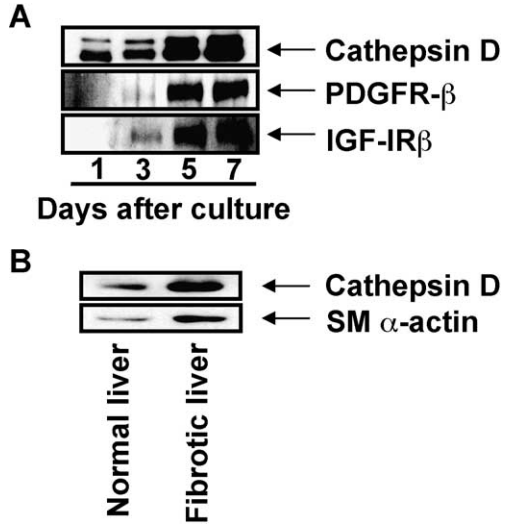

Fig. 3. Expression of cathepsin D in rat stellate cells. (A) Western blot showed that the level of cathepsin D (43 kDa) was induced in the course of stellate cell activation along with the expression of PDGF receptor β and IGF-I receptor β, well-known markers of cell activation. Typical results from five individual experiments are indicated. PDGFRβ, PDGF receptor β, IGF-IRβ, IGF-I receptor β. (B) Expression of cathepsin D protein was augmented in fibrotic liver tissue. Induction of cathepsin D protein coincided with the induction of smooth muscle α-actin in fibrotic liver. Typical results from three individual animals are indicated. SM α-actin, smooth muscle α-actin.

3.4. Expression of cathepsin D in activated stellate cells

Finally, we checked the expression of cathepsin D in stellate cells. As shown in Fig. 3A, cathepsin D (43 kDa) was induced in stellate cells in a similar way to the expression of PDGF receptor β and IGF-I receptor β. Western blot further revealed that cathepsin D was strongly expressed in fibrotic liver in comparison with normal liver.

4. Discussion

N-acetyl-L-cysteine is well-known as a glutathione precursor and as a scavenger of intracellular hydrogen peroxide, resulting in disruption of growth-stimulating intracellular signaling pathways and in cell cycle arrest. For instance, *N*-acetyl-L-cysteine was reported to inhibit tyrosine phosphorylation and the growth of vascular smooth muscle cells under PDGF-BB stimulation by scavenging hydrogen peroxide (Sundaresan et al., 1995). Several reports furthermore demonstrated that inhibition of MAPK and Akt activation by *N*-acetyl-L-cysteine was caused by its radical scavenging action. For instance, UV-induced phosphorylation of Akt at Ser-473 and Thr-308 in mouse epidermal Cl41 cells was attenuated by *N*-acetyl-L-cysteine through scavenging of H_2O_2 (Huang et al., 2001). The activation of MAPK in the p21(RAS)/Raf-1/MEK2 pathway in human aortic smooth muscle cells stimulated by lactosylceramide was reported to be inhibited by *N*-acetyl-L-cysteine through its radical scavenging action (Bhunia et al., 1997). However, in stellate cells, we hardly detected the generation of hydrogen peroxide even under stimulation with either IGF-I or PDGF-BB (data not shown). In this context, we speculated on other mechanisms by which *N*-acetyl-L-cysteine inhibited stellate cell growth. These included 1) *N*-acetyl-L-cysteine may suppress the generation of PDGF-BB and IGF-I by stellate cells, thereby interrupting an autocrine stimulatory loop for stellate cell proliferation, and 2) *N*-acetyl-L-cysteine may modify the expression of receptors for PDGF and IGF-I, resulting in the desensitization of stellate cells to these growth factors.

In the course of analysis, we found that pepstatin A was able to ameliorate the *N*-acetyl-L-cysteine-induced inhibition of stellate cell DNA synthesis. This indicated that proteolysis induced by cathepsin D, an aspartic acid protease, may be involved in the action of *N*-acetyl-L-cysteine. Indeed, we found here that cathepsin D was induced in stellate cells, in particular activated ones, and that cathepsin D in the presence of *N*-acetyl-L-cysteine modulated the level of PDGF receptor β protein and IGF-I receptor β protein in stellate cells. This is in line with a previous report showing that cathepsin B is involved in the pharmaceutical effect of *N*-acetyl-L-cysteine (Okuyama et al., 2001). Thus, proteolysis of PDGF receptor β and IGF-I receptor β in stellate cells induced by cathepsin B and cathepsin D, at least under

reducing conditions, may play a pivotal role in the cellular response to growth factors. In vivo, the activity of these cathepsins may be tightly controlled by endogenous reducers such as glutathione, which is abundantly produced and secreted by hepatocytes, neighbors of stellate cells.

The involvement of cathepsins in hepatic pathophysiology has never been well-characterized. Recent investigations demonstrated that cathepsin B plays a profound role in the tumor necrosis factor-α-mediated apoptosis of hepatocytes, by promoting mitochondrial release of cytochrome c and activation of caspases 9 and 3 (Guicciardi et al., 2000, 2001). Cathepsin B was also reported to be involved in hepatocyte apoptosis induced by toxic bile salt (Faubion et al., 1999). In a rodent model, increased activities of liver cathepsins B, D and T, and fibrosis were observed in carbon tetrachloride-treated rats, indicating the profound role of cathepsins in matrix turnover (Yamamoto et al., 1992). Clinically, serum levels of cathepsins B, D and L are elevated in patients with cirrhosis and hepatic carcinoma (Brouillet et al., 1991; Leto et al., 1996, 1997). Despite these observations, detailed molecular mechanisms of cathepsins in hepatic fibrosis are lacking so far.

The present investigation demonstrated that cathepsin D is a new marker for activated stellate cells. Smooth muscle α-actin and PDGF receptor β have been most commonly used as indicators of cell activation. However, recent analysis has revealed that neural cell-adhesion molecule, prion-related protein, and intercellular adhesion molecule-1 can also be used (Friedman, 1993, 2000; Kawada, 1997; Pinzani and Marra, 2001; Rockey, 2001). More recently, we discovered a novel protein induced in activated stellate cells and termed it stellate cell activation-associated protein (STAP) (Kawada et al., 2001). The finding of these molecules closely associated with stellate cell activation contributes to the analysis of the state of stellate cells, to the search for agents that modulate their function, and to the pathological diagnosis of liver fibrosis.

In summary, the present investigation revealed that (1) *N*-acetyl-L-cysteine effectively downregulates the level of PDGF receptor β protein and IGF-I receptor β protein, thereby hampering growth-stimulating intracellular signaling pathways in stellate cells. (2) Pepstatin A restores the response of stellate cells to PDGF-BB and IGF-I even in the presence of *N*-acetyl-L-cysteine, revealing the involvement of cathepsin D in the pharmaceutical action of *N*-acetyl-L-cysteine. Because such an effect of *N*-acetyl-L-cysteine is independent of its radical scavenging action, the present results shed new light on the molecular action of *N*-acetyl-L-cysteine.

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