Effect of N-Acetylcysteine on the Matrix Metalloproteinases and Their Inhibitors in Carbon Tetrachloride-Induced Hepatotoxicity

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Abstract

Matrix metalloproteinases (MMPs) are a group of zinc proteases that serve the function of breaking down extracellular matrix (ECM). The present study evaluated the role of N-acetylcysteine (NAC) on the increased deposition of ECM in hepatic and glomerular fibrosis caused by carbon tetrachloride (CCl₄). The activity of MMPs increased and the levels of tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) decreased in the liver and kidney of CCl₄-treated rats. Rats treated with CCl₄ and NAC showed increased activities of MMPs and decreased levels of TIMP-1 and TIMP-2 in the liver and kidney. Treatment with NAC resulted in the effective degradation of ECM due to an increase in the activities of MMPs and a decrease in the levels of TIMPs.

Key words: matrix metalloproteinases, carbon tetrachloride, N-acetylcysteine, Wistar rats, liver, kidney

INTRODUCTION

Carbon tetrachloride (CCl₄)-induced rats are highly useful as experimental models for the study of certain hepatotoxic effects (1). CCl₄ at low quantity is responsible for the appearance of reversible lesions in the liver that occur in all subjects exposed at certain doses of the substance (2). CCl₄ metabolism begins with the formation of the trichloromethyl free radical, CCl₃· through the action of the mixed function cytochrome P450 oxygenase system of the endoplasmic reticulum (3). The CCl₃· radical reacts with various biologically important substances such as amino acids, nucleotides, and fatty acids, as well as proteins, nucleic acids, and lipids (4). In the presence of oxygen, the CCl₃· radical is converted to the trichloromethyl peroxy radical (CCl₃OO·). This radical is more reactive and is capable of abstracting a hydrogen from polyunsaturated fatty acids (PUFA) and initiates the process of lipid peroxidation (5).

Extracellular matrix (ECM) plays an important role in the regulation of cell function and changes in its composition and structure could have a profound patho-physiological implication (6). Various types of collagens, laminin, fibronectin, and sulfated proteoglycans are the normal components of glomerular matrix (7). Excessive accumulation of the ECM proteins and modification of the liver architecture are major patho-histological features of the liver disease progression and are closely related to the action of MMPs (8). An imbalance between

synthesis and degradation of these matrix components is closely associated with the accumulation of ECM within the glomerulus and subsequent progression of renal diseases (9).

Extracellular matrix degradation is controlled primarily by matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent proteases that degrade ECM proteins and are essential for cellular migration and tissue remodeling under physiological and pathological conditions (10). MMPs are synthesized as latent pro-enzymes, which are later activated by serine proteases, including trypsin and plasmin, active MMP-2 and membrane type-MMPs (11). The activity of MMPs is tightly regulated by tissue inhibitors of MMPs (TIMPs). TIMPs are 21~28 kD proteins with a highly conserved sequence of six intramolecular disulfide bonds (12). TIMPs form physiologically irreversible complexes with all types of activated MMPs (13). MMPs have been proposed as important factors driving fibrosis in the liver and glomerulus. MMP-2 and MMP-9 belong to the type IV collagenase family and are responsible for the degradation of collagen type IV, V, VII, elastin, and fibronectin (14). Antioxidant compounds have been shown beneficial on matrix remodeling by maintaining MMPs/TIMPs equilibrium in hepatic fibrosis (15,16).

N-acetylcysteine (NAC) contains free sulfhydryl groups and it may directly react with electrophilic compounds such as free radicals (17). Miesel and Zuber (18) have reported that NAC contributes significantly to the

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intracellular antioxidant defense system by acting as a powerful consumer of superoxide, singlet oxygen and hydroxyl radicals. NAC could significantly interfere with the pathophysiology of free radical producing drug induced oxidative stress (19). Several reports have shown that NAC treatment protects against acetaminophen hepatotoxicity in patients (20) and in rats (21,22).

The purpose of the present study is to assess the impact of NAC on the activities of MMPs (MMP-2 and MMP-9) and the levels of their inhibitors (TIMP-1 and TIMP-2) in liver and kidney in CCl₄-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Experimental animals

Male albino Wistar rats of body weight 150~180 g were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University and were maintained there. The rats were housed in polypropylene cages lined with husk. They were fed on a standard pellet diet (Agro Corporation Private Ltd., Bangalore, India) and water *ad libitum*.

Chemicals

N-acetyl cysteine, tris, acrylamide, bis-acrylamide, gelatin, sodium dodecyl sulphate (SDS), ammonium persulfate, o-dianisidine, and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were obtained from Sigma Chemical Company (St. Louis, MO, USA). CCl₄ was purchased from Merck Ltd. (Mumbai, India). All other chemicals used in our study were of high analytical grade.

Experimental design

In our study, a total of 24 rats were used. The rats were divided into 4 groups of 6 rats each. NAC was orally administered to rats daily at a dose of 150 mg/kg body weight for three months using an intragastric tube. CCl₄ was subcutaneously injected to rats at a dose of 3 mL/kg body weight/week for three months.

Group I	Normal control rats
Group II	Normal rats orally administered with NAC (150 mg/kg body weight/daily) (23)
Group III	Rats subcutaneously injected with CCl ₄ (3 mL/kg body weight/week) (24)
Group IV	Rats orally administered with NAC (150 mg/kg body weight/daily) along with subcutaneous injection of CCl ₄ (3 mL/kg body weight/week).

The experiment was carried out for a period of three months. All the experimental protocols were approved

by the Ethical Committee of Annamalai University. After the last treatment, the animals were fasted overnight and sacrificed by cervical dislocation.

Tissue preparation

Liver and kidney were dissected out immediately from rats, cleared off blood and stored in ice-cold containers containing phosphate buffered saline (PBS) with protease inhibitors. The tissues were washed with PBS and weighed. A known amount of the tissue was homogenized with 0.9% NaCl and the homogenate was used for estimation of MMPs and TIMPs.

Biochemical estimations

Estimation of MMPs: The total activities of MMPs were assessed by multiwell zymography as described by Sudhakaran and Ambili (25). About 50~100 µL of the sample was added to the wells of a multiwell plate. Gelatin was added to standard Laemmli acrylamide polymerization mixture, at a final concentration of 1.0 mg/mL under non-reducing conditions. This was added quickly into each well and allowed to set. After the gel was polymerized, the gel gently was detached from the bottom of the well and incubated with Tris-HCl solution (0.05 M, pH 7.0/5 mM CaCl₂) for 36 hr. Each gel bit was then stained using coomassie blue and destained in water. The activity was determined using a laser densitometer.

Individual MMP expression was analyzed by gelatin zymography (26). Gelatin was added to standard Laemmli acrylamide polymerization mixture at a final concentration of 1.0 mg/mL under non-reducing condition. Concentrated tissue extract was mixed with 3:1 with substrate gel sample buffer and 20~30 µL were loaded immediately into Laemmli acrylamide stacking gel on a mini gel. Gels were run at 15 mA/gel while stacking and at 20 mA/gel during the separating phase at 4°C. Following electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking at room temperature for 30 min with one change of detergent solution. Gel was rinsed and incubated overnight at 37°C in Tris/HCl (0.05 M, pH 7.5/5 mM CaCl₂). The gel was then stained using Coomassie blue and destained in water. The activity was determined using a laser densitometer.

MMP-2 and MMP-9 were estimated by succinylation method described by Baragi et al. (27). Densitometric analysis was carried out using Gel/Chemi Doc, Bio-Rad software 4.4.1. Gelatin was dissolved in 50 mM borate buffer (pH 8.5) at a concentration of 20 mg/mL. An equal amount of succinic anhydride was then gradually added to the solution and the pH of the reaction was maintained at 8.0 to 8.5 by the addition of 1.0 M NaOH.

The succinylated gelatin was then dialyzed extensively against 50 mM sodium borate buffer, pH 8.5. All assays were done in 96 well flat bottom microtitre plates. MMP-2 was assayed in 50 mM borate buffer (pH 7.0) with 10 mM CaCl_2 , whereas MMP-9 was assayed in 50 mM borate buffer (pH 8.5). The total reaction volume was 150 μL which contained enzyme and 200 μg of succinylated gelatin. Blank without substrate but with appropriate buffer and enzyme was performed for each enzyme assay. The reaction was carried out at 37°C for 30 min. Fifty μL of 0.3% TNBS was then added to the reaction mixture and incubated at room temperature for 20 min. The optical density was determined at 420 nm. The activities of MMP-2 and MMP-9 are expressed as the absorbance at 420 nm/min/mg protein.

Estimation of TIMPs: The levels of TIMPs were estimated by ELISA method as described by Iype et al. (28). 100 μL of tissue homogenate was coated in different wells in 24 well ELISA plate. They were incubated at room temperature for 3 hr. After incubation, the wells were drained and washed with PBS twice. Then added 200 μL of 0.05% tween-20 and incubated at room temperature for 1 hr. It was washed with 0.05% tween-20 in PBS twice. Added 100 μL of primary antibody and incubated at room temperature for 2 hr. It was washed in tween-20 twice. Then added secondary antibody coupled to horseradish peroxidase and kept at room temperature for 1 hr. It was washed again with tween-PBS followed by PBS twice. Then added 1.0 mL of o-dianisidine to all the wells and incubated in dark for 30 min. The reaction was arrested by adding 5 N HCl and the optical density was measured at 450 nm. The levels of TIMP-1 and TIMP-2 were expressed as the absorbance at 450 nm/min/mg protein.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. The values are mean of 6 rats for each group and $p < 0.05$ was considered as being statistically significant.

RESULTS

The total MMP activities of liver and kidney samples in rats treated with CCl_4 and with NAC are shown in Fig. 1 and 2 and the densitometric readings are shown in Fig. 1a and 2a. In CCl_4 -treated rats, the total MMP activities were decreased. Administration of NAC to CCl_4 -treated rats increased the MMP activities. In normal+NAC treated rats, a normal pattern of multiwell zymography and relative densitometric reading was

Fig. 1. Zymogram of liver samples during NAC treatment in CCl_4 toxicity.

Fig. 1a. Densitometry of multiwell zymogram of liver samples during NAC treatment in CCl_4 toxicity.

Fig. 2. Zymogram of kidney samples during NAC treatment in CCl_4 toxicity.

Fig. 2a. Densitometry of multiwell zymogram of kidney samples during NAC treatment in CCl_4 toxicity.

Fig. 3. Zymogram of liver samples during NAC treatment in CCl_4 toxicity.

Fig. 3a. Densitometry of multiwell zymogram of liver samples during NAC treatment in CCl_4 toxicity.

Fig. 4. Zymogram of kidney samples during NAC treatment in CCl_4 toxicity.

obtained.

Gelatin zymograms of liver and kidney samples from rats treated with CCl_4 and with NAC are shown in Fig. 3 and 4, respectively. Four different types of MMPs (130 kD, 92 kD, 72 kD, and 45 kD) were expressed in liver and three different types of MMPs (130 kD, 92 kD, and 72 kD) were expressed in kidney of rats, during CCl_4 administration and subsequent treatment with NAC. The densitometric reading of the individual MMPs are given in Fig. 3a and 4a, respectively. The activities of MMPs were decreased in CCl_4 -treated rats. When these rats were treated with NAC, there was an increase in the MMP activities.

The levels of TIMPs (TIMP-1 and TIMP-2) in liver

Fig. 4a. Densitometry of multiwell zymogram of kidney samples during NAC treatment in CCl_4 toxicity.

TIMP-1

TIMP-2

Fig. 5. Levels of TIMPs in liver of NAC and CCl_4 -treated rats. Each column is mean $\pm$ SD for 6 rats in each group. Columns that have a different letter (a-c) differ significantly with each other ($p < 0.05$, DMRT).

TIMP-1

TIMP-2

Fig. 6. Levels of TIMPs in kidney of NAC and CCl_4 -treated rats. Each column is mean $\pm$ SD for 6 rats in each group. Columns that have a different letter (a-c) differ significantly with each other ($p < 0.05$, DMRT).

and kidney of normal and experimental groups are shown in Fig. 5 and 6, respectively. A significant increase in the levels of TIMPs was observed in the liver and kidney of rats treated with CCl₄. When NAC was administered, their levels decreased significantly. When normal rats were treated with NAC, there was no significant change in the levels of TIMPs.

DISCUSSION

Hepatic stellate cells (HSCs) are located in the hepatic perisinusoidal spaces (29) and are responsible for excess collagen deposition, fibrosis and cirrhosis in liver injury. Liver injury is associated with activation of these hepatic stellate cells (30). HSCs proliferate and transform to enlarged myofibroblast cells, and these activated HSCs are considered to be the major source of collagenous and non-collagenous matrix proteins which accumulate in fibrotic liver (31). There is an imbalance between synthesis and degradation of matrix components such as collagens, laminin, fibronectin and sulfated proteoglycans in various types of renal diseases and it is closely associated with accumulation of ECM during its progression (32). The progression of renal diseases due to structural remodeling has also been shown to be associated with an imbalance between synthesis and degradation of MMPs/TIMPs (33).

MMPs are a family of proteolytic enzymes that share several structural and functional characteristics, but have different substrate specificities (34). MMP-2 (92 kD) is predominantly secreted by endothelial cells and MMP-9 (72 kD) is the major MMP secreted from macrophages (35). MMP-2 and MMP-9 are often detected by gelatin zymography because of their high-gelatinolytic activity (36). These two gelatinases (MMP-2 and MMP-9) have a gelatin-binding domain inserted between the catalytic domain and active site domain (12).

A decrease in the activities of MMPs in the liver and kidney of rats treated with CCl₄ was observed in this study. Treatment with CCl₄ generates free radicals that trigger a cascade of events resulting in fibrosis mimicking the oxidative stress that has a fibrogenic effect on hepatic stellate cells (HSCs) (37). Increased ROS production may also be involved in stimulation of excessive matrix production in vivo (38). Matrix metalloproteinases target the various substrates of the ECM, causing their dissolution (39). A decrease in the activities of MMPs in CCl₄-treated group signifies end stage fibrosis. A decrease in the MMP activities in progressive stage of liver fibrosis has been reported (8). This decrease could be due to decreased procollagen gene expression and biosynthesis, decreased activation of proMMPs or specific

inhibition of native MMPs (8).

The expression and ratio of MMPs/TIMPs are key factors to ECM remodeling (40). TIMP-1 is produced by kupffer cells, HSC and myofibroblasts in liver, but most of it is mainly produced by activated HSCs. TIMP-2 is highly expressed in hepatocytes and kupffer cells, but the main origin is activated HSCs (40). TIMPs are known to bind tightly to particular latent MMPs: TIMP-1 binds tightly to proMMP-9 and TIMP-2 binds tightly to proMMP-2 (12). TIMP-1 could inhibit most of the MMPs by integrating them at a ratio of 1:1 to form a complex. The ability of TIMP-2 to integrated MMP-2 is 7~9 fold higher than that of TIMP-1 (40). In CCl₄-induced liver fibrosis in rats, an increased expression of TIMPs was observed by us and has also been reported by other researchers (30,41). CCl₄ by producing a large amount of ROS enhances TIMPs generation, which in turn favored collagen deposition by inhibiting MMP activities (42).

Oxidative stress has an important role in the pathogenesis of liver and glomerular fibrosis and decreasing the production of ROS can prevent excess matrix deposition in liver and kidney. NAC administration to CCl₄-treated rats decreased the excessive collagen deposition in the matrix by increasing MMP activities and by decreasing the levels of TIMPs in the liver and kidney. NAC could significantly interfere with the pathophysiology of free radical producing drug induced oxidative stress (19). The result is decreased lipid peroxidation and prevention of liver and kidney damage, progressing towards end fibrotic stage. In addition, the protective effect of some antioxidant compounds on matrix remodeling by maintaining MMPs/TIMPs equilibrium have been reported (15,16).

The overall results of our study confirm the protective effect of NAC in CCl₄-induced toxicity in rats by its ability of free radical scavenging and antioxidant properties. NAC exhibits its antifibrogenic activity by increasing MMP activities and decreasing the levels of TIMPs in the liver and kidney of CCl₄-treated rats.

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