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Observation of Esterase-Like-Albumin Activity during *N'*-Nitrosodimethyl amine Induced Hepatic Fibrosis in a Mammalian Model

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Abstract

Aim: Hepatic fibrosis (HF) is characterized by irregular growth and amassing of fibrous scar tissues in the liver causing weakened hepatocytes metabolism and protein level alterations, including albumin. Albumin with M_r~68-70 kDa is unglycosylated soluble plasma protein with various biological roles. In this study, we demonstrate 'esterase-like activity' of albumin during NDMA-induced HF in rats.

Material and Methods: In rats, HF was induced by weekly i.p. injections of NDMA in doses of 10 mg/kg b.wt. Sera of controls (untreated) and treated rats were processed for biochemical tests, electrophoretic profiling and in-gel esterase activity localization using α, β-naphthyl acetates. H&E staining of liver sections (~ 5 μm) was done to confirm induction of HF.

Results: NDMA satisfactorily induces hepatic fibrosis within 21 days which is also evident by significant increase in SALP, SGOT, SGPT and bilirubin levels in rats. 'Esterase-like activity' of albumin detected in animal sera remains stable throughout the course of treatment irrespective of other biochemical changes.

Conclusion: During pathogenesis of HF, formation of stable esterase-albumin complex may have some important role and hence, prior recommending the use of albumin as diagnostic marker we propose further investigations to elucidate the mechanism of its formation.

Introduction

Albumin is quantitatively the most important soluble plasma protein. It plays various important biological roles including maintenance of plasma oncotic pressure [1]. Due to its ability to bind reversibly, it act as a transporter protein for many endogenous and exogenous compounds including various hormones, non-steroidal anti-inflammatory drugs; maintenance of intravascular volume and capillary permeability, removal of potentially toxic compounds and dissociation of free radicals, along with neutrophil activation and adhesion [2-4]. Serum albumin is a 66.5 kDa (70 kDa in case of rat) unglycosylated, single chain polypeptide, made up of 585 amino acids, arranged in three homologous α-helical domains, each domain comprising of subdomain A and B, consisting of six and four alpha-helices, respectively [5]. In addition to several other sites [6-7], the domain II and III of HSA contain two primary drug binding sites: Sudlow's site I and site II [8].

Serum albumin level is an important predictive

marker in advanced liver diseases. Liver diseases often results in the alteration of both structure and function of albumin: hypoalbuminemia due to reduced synthesis, oxidative modification in its structure and binding of bilirubin to its active sites etc. In addition to the above roles, another significant functional property of albumins is its catalytic activity toward a broad range of molecules like esters, amides, and phosphates etc [9-10].

Albumin has been reported to exhibit esterase-like catalytic activity, although this hydrolysis is stereo selective [9-14]. HSA-catalyzed hydrolysis of *p*-nitrophenol esters was first reported in 1951 [15]. This "esterase-like" activity of HSA has been attributed to the sub-domain IIIA [13], in addition to this, nuclear magnetic resonance (NMR) spectroscopic studies reported that the hydrolysis rates of aspirin were greatly enhanced in the presence of HSA, thus confirming the esterase-like activity of subdomain IIA of HSA (site I) [16-17]. Site-directed mutagenesis studies have confirmed Arg-410 and Tyr-411 are indeed important for the esterase activity of HSA [18].

'Esterase-like-albumin' activity has been reported in many clinical conditions with an emphasis on its prognostic value in the diagnosis or pathogenesis of the disease [19]. However, such activity of albumin in patients with osteoarthritis has also been demonstrated previously with no implications in the pathogenesis of the disease [20]. During the present investigations, we demonstrate "esterase-like-albumin" activity in a mammalian model during the progression of *N'*-Nitrosodimethyl amine (NDMA) induced hepatic fibrosis.

Material and Methods

Chemicals and reagents

Acrylamide, Bis-acrylamide, Ammonium persulphate (APS), TEMED, *N'*-Nitrosodimethyl amine (NDMA) were purchased from Sigma-Aldrich. Tris buffer, CBBR-250, Hematoxylin and eosin stains and Bovine serum albumin (BSA) were obtained from SRL, India and the chemicals used for in-gel esterase staining including α- and β-naphthylacetate, Fast blue RR were purchased from Qualigens Fine Chemicals, India. All other chemicals and reagents used were of analytical grade.

Care and maintenance of animals

A total number of twenty four adult healthy male Wistar rats, weighing around 145 ± 10g were used in the present study. The rats were housed in well aerated polycarbonate cages with proper humane care and were fed regularly with sterilized diet and water available *ad libitum* with light: dark exposure of 12:12 hours. Prior treatment, the animals were acclimatized for about a week in laboratory conditions.

Induction of hepatic fibrosis

The animals (n=24) were divided into two groups comprising of twelve rats each. One group received intraperitoneal injections of *N'*-Nitrosodimethyl amine (NDMA) in doses of 10 mg kg^{-1} body weight (diluted with 0.15M sterile NaCl) and the other group served as control and received same amount of sterile NaCl through intraperitoneal injections without anesthesia. The injections were given as described earlier [21].

Histological assessment of hepatic fibrosis

The degree of hepatic fibrosis was monitored by Hematoxylin and Eosin (H&E) staining of serial sections of liver. Stained slides were examined and photographed under Nikon microscope with an LCD attachment (Model: 80*i*).

Sera collection and protein estimation

Blood samples were collected through cardiac puncture on weekly basis i.e. on Days 7, 14 and 21 from the onset of experiment. Appropriate controls were also maintained and their blood samples were taken on the same days. Blood samples were kept for 1-2 hr to ooze out the sera. Samples were centrifuged at 3000 rpm and 4°C for 5-10 min. The resulting supernatant (sera) was either analyzed afresh or stored in different aliquots at -20 °C for further analysis.

Protein concentration of the sera samples were determined by the method described earlier using bovine serum albumin (BSA) as the standard [22]. Absorbance was taken at 540 nm on a UV-1700 Pharma-spec UV-Visible spectrophotometer. Some marker proteins of liver function test (LFT) like sera alkaline phosphatase (SALP), glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), albumin and bilirubin were estimated using commercial kits supplied by Coral and AutoZyme diagnostics Ltd. (India).

In-gel enzyme activity localization

Vertical slab non-denaturing 7.5%

polyacrylamide gel (80 x 60 x 1 mm) electrophoresis (PAGE) of sera samples collected at days 7, 14 and 21 was carried out (in replicate) according to the protocol of Laemmli [23] with a slight modification that the gels were lacking SDS. Parallel controls were also run in the similar conditions. Equal quantities of protein (8 μg) were loaded in each well. Electrophoretic runs were made for 5 hrs at room temperature, stained with Coomassie Brilliant Blue (CBB-R250) and destained overnight in 7% glacial acetic acid. Replica gels were processed for in-gel esterases activity localization as previously described [24]. Briefly, the gels were incubated in a reaction mixture containing α, β-naphthyl acetate (5.58 x 10^{-3} mM, pH 7.5) as substrates along with dye coupler Fast Blue RR at 25°C in dark. Upon development of visible bands of esterase activity, the reaction was stopped by fixing the gels in 7% glacial acetic acid for 20 min, followed by preservation of the gel in 5% acetic acid.

Molecular weight estimation

Following protein estimation, sera samples were immediately mixed in evaluated amounts of 4x sample buffer. The samples contained the final concentrations of 0.0625M Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 5% glycerol and 0.001% Bromophenol blue. They were incubated in boiling water bath for 3-5 min and loaded in equal amounts on 10% SDS-PA gels as per standard protocol [23]. Visualization of bands was done by Coomassie Brilliant Blue (CBB) following overnight washing of gels in 5% acetic acid. Molecular weight of polypeptides was estimated using chicken actomyosin as molecular weight (M_r) marker. Polypeptides in chicken actomyosin ranges from 200 kDa to 15 kDa, consisting of myosin heavy chain (200 kDa), actin (46 kDa), troponin (42 kDa), tropomyosin (39 kDa) and several light chains of low molecular weights (23, 18, 17 and 15 kDa).

Densitometry and quantitative assessment of PAGE profiles

Stained PA-gels were photographed using SONY-CYBERSHOT digital camera (Zoom-4X, 14.1 Mega pixels) and by direct scanning on an all-in-one HP Deskjet (F370) computer assembly. Gel-scans were processed through Adobe Photoshop (version 7.0; Windows XP) to obtain the best contrast for densitometric analysis through Scion Imaging (Scion Corporation; Beta release, 4.0). Molecular weight was estimated using GelPro (Media Cybernetics, USA) software programs.

Results

Treatment of rats with *N'*-Nitrosodimethyl amine successfully induces hepatic fibrosis (HF) in a way similar to humans. Induced hepatic fibrosis in rats is a very successful and reproducible model to study various biochemical and molecular aspects. During HF abnormal,

Table 1: Liver function test (LFT) biomarkers in the sera of control and treated rats. Treatment with *N'*-Nitrosodimethyl amine (NDMA) in doses of 10 mg kg⁻¹ body weight was given to animals for 21 days under laboratory conditions. [Values are expressed as mean ± s.d.]

Biochemical markers	Control	Treated
SALP (U/L)	313 ± 26.12	691 ± 31.19[*]
SGOT (U/L)	131 ± 17.11	188 ± 18.66[*]
SGPT (U/L)	52 ± 6.34	69 ± 3.66[*]
TBIL (mg/dl)	0.307 ± 0.02	1.31 ± 0.11[**]
DBIL (mg/dl)	0.14 ± 0.01	0.155 ± 0.06
TSP (g/dl)	7.0 ± 0.59	6.26 ± 0.99
SAlb (g/dl)	3.12 ± 0.18	3.81 ± 0.45

SALP = Sera alkaline phosphatase; SGOT = Sera glutamic oxaloacetic transaminase; SGPT = Sera glutamic pyruvic transaminase; TBIL = Total bilirubin; DBIL = Direct bilirubin; TSP = total sera proteins; SAlb = Sera albumin. [*]$P<0.05$; [**]$P<0.001$.

tough and non-functional fibrous connective tissue, especially mature collagen fibers deposit in the extracellular matrix (ECM). H & E staining of liver biopsies of fibrotic rats showed disruption in typical liver architecture, severe congestion and hemorrhage at day 7, followed by severe neutrophilic infiltration and appearance of patchy necrotic areas by day 14 and
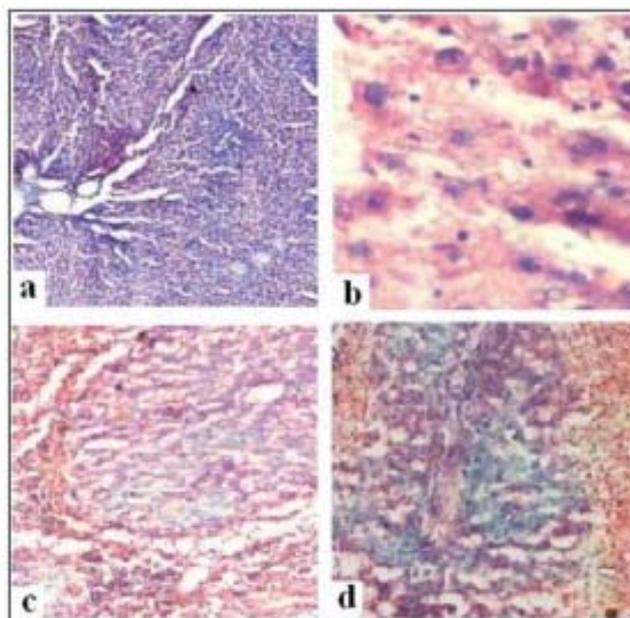

Figure 1: Hematoxylin and Eosin (H & E) staining of liver sections demonstrate induction of hepatic fibrosis in rats. (a) Control liver (×40), (b) Day-7 (×100), (c) Day-14 (×100), (d) Day-21 (×100).
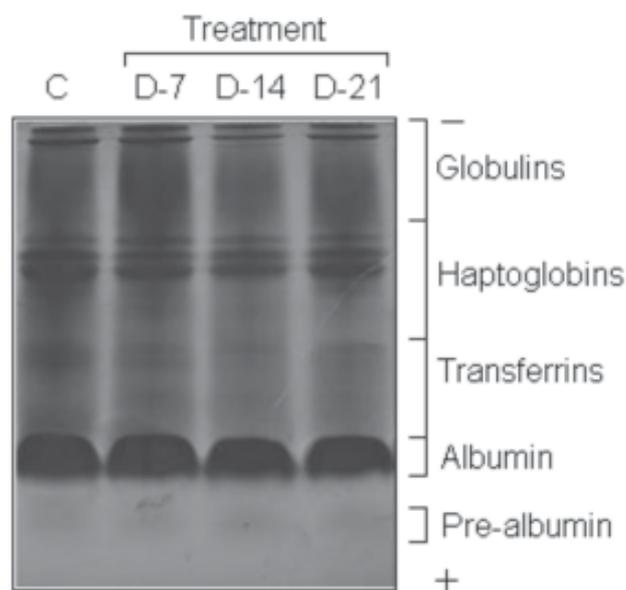
Figure 2: *Typical sera PAGE profiles of Control (C) and fibrotic rats at days-7, -14 and -21. The main sera fractions are labeled in the figure. Electrophoretic runs were made in the absence of SDS.*

Figure 3: *Densitograms of representative sera protein profiles run under denatured conditions (SDS-PAGE). Human sera (a) and, rat sera in control (b) and day-21 NDMA treated severely fibrotic rats (c). Arrow in the PAGE lanes show major band while in the densitogram show corresponding peak of albumin.*

fibrosis with clear accumulation of collagen fibers by the end of 21 days of NDMA treatment (Figure 1). The analyses of liver function test biochemical markers in the sera of NDMA treated animals showed significant rise in the levels of sera alkaline phosphatases (SALP), glutamic oxaloacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) compared with controls. An increase of the order of ~ 120, ~ 44 and ~ 33% was noted for SALP, SGOT and SGPT respectively in treated animals (Table 1).

Native PAGE profiles of sera samples of fibrotic rats revealed minor differences compared with control. However, typical sera protein fractions of globulins, haptoglobins, transferrins and albumin barely show any difference. Albumin as the major sera fraction stacks at the anodal side was identified by routine Coomassie Blue staining (Figure 2). Representative sera profiles in SDS-PAGE showed the existence of albumin in a molecular weight range similar to HSA. GelPro analysis of gel-scans revealed sera albumin of rat with a molecular weight of ~ 66 kDa (Figure 3).

Esterase-like activity of sera albumin was demonstrated by histochemical staining using α, β-naphthyl acetates as substrates. The activity of enzyme was satisfactory at pH, 7.5 in the dark at 25 °C. In the sera of control rats, esterase-like activity of albumin was detected in anodal region at a position similar to albumin
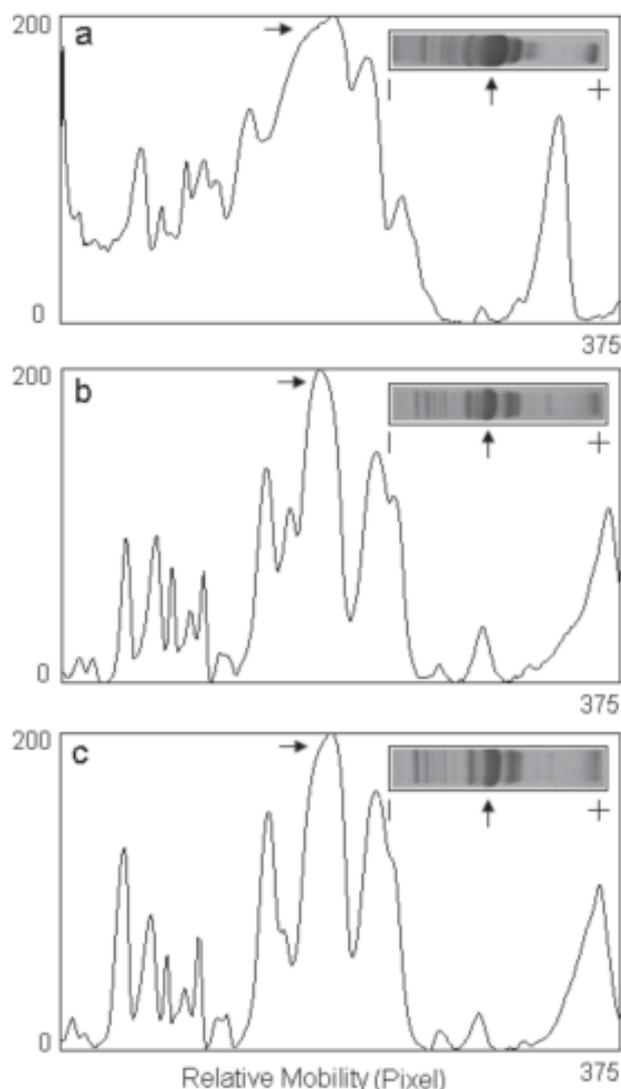
(Figure 4). Enzyme activity of albumin persists up to 21 days indicating active enzyme-protein complex irrespective of other biochemical disturbances during the progression of disease.

Discussion

Hepatic fibrosis (HF) is a pathological state that arises due to loss in equilibrium between the synthesis (fibrogenesis) and breakdown (fibrolysis) of matrix tissue. This leads to extensive scar tissue formation and deposition under normal physiological conditions [25-
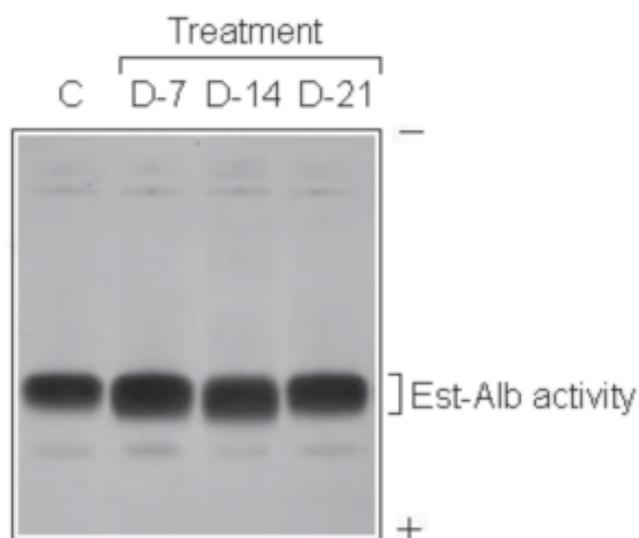
Figure 4: Sera PAGE profiles of fibrotic rats run under native conditions showing the presence of 'esterase-like activity' of albumin in control (C), and progressively fibrotic stages of rats.

26]. Injury to liver triggers this fundamental process of tough tissue formation. The disease is now a raising concern in almost all parts of the world due to increased hepatitis viral infections. In United States, Europe and Japan, hepatitis C viral (HCV) infection and alcohol have been the major causes of HF. Whereas in China, Hepatitis B viral (HBV) infection is the main cause [27-30]. In fact in USA and Europe, liver cirrhosis seems to be the most common non-neoplastic cause of death among hepatobiliary and digestive diseases [31]. In India, alcohol consumption and nonalcoholic fatty liver disease (NAFLD) have been suggested to be the key cause of HF. Considering the events of death all over the world, it becomes necessary to understand the biochemical and molecular mechanism of the disease. Some interesting literature published in the recent past signifies the importance of understanding the mechanism of the disease [25-26]. As such, to study the underlying mechanism of HF in humans is difficult due to ethical considerations; therefore alternate models of the disease may suffice to investigate different aspects of the disease. During the present study, we have satisfactorily induced hepatic fibrosis in rats using *N'*-Nitrosodimethyl amine (NDMA). All the reported classical features of the disease [24, 32] were noted in the H & E stained liver biopsies of fibrotic rats with confirmed inflammation, disruption in typical liver design, hemorrhage and distinctive collagenesis (Figure 1). More importantly, our quantitative data on LFT parameters demonstrates induced hepatotoxicity in experimental animals within 21 days of NDMA treatment in doses of 10 mg kg^{-1} b.wt. Induction

of hepatic fibrosis is strongly supported by numerical data obtained for sera alkaline phosphatases (SALP), glutamic oxaloacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) and bilirubin, which showed significant rise in their levels (Table 1).

Scarce reports are available on the sera profiling of fibrotic rats [32-34]. In the present study, main activity fractions of sera detected on native PAGE were globulins, haptoglobins, transferrins and albumin which have already been demonstrated in rats and human [28, 34-35]. The major activity band remains that of albumin in control as well as in fibrotic animals. Actually, albumin is the only molecule which in addition to its ligand binding ability, has been reported to show hydrolytic 'esterase-like activity' with various compounds [9, 12]. These include p-nitrophenyl acetate [36], phenylacetate, α-naphthyl acetate [37], β-naphthyl acetate [38], asprin [39], o-nitrotrifluoroacetanilide [40], nicotinate esters [12], long and short chain fatty acid [41], o-nitroacetanilide [42], organophosphorus pesticides [43] and they result in the acetylation of tyrosine residue at 411 [13]. This 'esterase-like activity' of serum albumin is more prominent in primates than in other group of animals [44], being highest in humans and absent in horse serum [45-46].

Albumin as a main activity in the sera of rat stacks as a band of molecular weight of ~ 66 kDa on denatured PA gels which is in agreement with reported literature [32-34]. 'Esterase-like albumin' activity in fibrotic rats is demonstrated using α, β naphthyl acetate as substrate (5.58 x 10^{-3} mM) and dye-coupler fast blue RR (pH, 7.5). Following incubation of native sera gels in reaction mixture for esterase activity localization, along with the normal serum esterases, albumin also takes strong staining with α, β naphthyl acetate. This indicates that albumin retains its 'esterase-like activity' in normal as well as in treated rats. The mechanism of formation of this esterase-albumin complex is well understood, however the existence of this complex during the progression of hepatic fibrosis has not been documented so far. Previously, levels of albumin have been suggested to use as biomarker for the assessment of hepatic fibrosis [47-49], but the existence of esterase-albumin complex in the fibrotic rats demands appropriate revisions in some of the published reports where albumin has been used as indicative biomarker of disease development. Therefore, while recommending the use of albumin as a diagnostic marker, we propose further investigations to elucidate the mechanism of esterase-albumin complex formation during the progression of hepatic fibrosis.

Acknowledgements

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