Herbal Supplement Attenuation of Cardiac Fibrosis in Rats with CCl₄-Induced Liver Cirrhosis

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Abstract

Previously we found carbon tetrachloride (CCl₄) induced cirrhosis associated cardiac hypertrophy and apoptosis. The purpose of this study is to determine whether further CCl₄ treatment would induce cardiac cell fibrosis. The cardiac tissues were analyzed by H&E. histological staining, Trichrome Masson staining and Western blotting. The results showed that the CCl₄-treated-only group exhibits more trichrome staining, meaning that more fibrosis is present. Moreover, CCl₄ could further induce cardiac-fibrosis via TGF-β-p-Smad2/3-CTGF pathway. However, our data showed that the CCl₄-induced cardiac abnormalities were attenuated by Ocimum gratissimum extract (OGE) and silymarin co-treatments. In conclusion, our results indicated that the OGE and silymarin may be a potential traditional herb for the protection of cardiac tissues from the CCl₄ induced cirrhosis associated cardiac fibrosis through modulating the TGF-β signaling pathway.

Key Words: cardiac fibrosis, CCl₄, Ocimum gratissimum, silymarin, TGF-β

Introduction

Advanced cirrhosis has consistently been diagnosed with increased cardiac output and reduced systemic vascular resistance, which are typical of cardiac hyperdynamic circulation (12, 18, 22, 41). Even though cirrhosis has long been clinically associated to cardiac dysfunction (42), how cirrhosis...
alters the cardiac structure at the molecular level is scarcely understood.

Cirrhosis is known to be associated with the activation of angiotensin II (Ang II) (14), which is also a factor in inducing cardiac hypertrophy, along with its downstream signaling pathways such as mitogen-activated protein kinases (MAPKs) and calcineurin that increase related gene expressions such as proto-oncogenes c-Fos and c-JUN, genes which encode atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), and structural genes β-myosin heavy chain (β-MHC) and skeletal α-actin (37). A proinflammatory cytokine associated with Ang II expression is interleukin-6 (IL-6) (40), which is an effective stimulator of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in cardiac hypertrophy (37). Nevertheless, the role of these protein markers and transcriptional factors in cardiac hypertrophy and remodeling in vivo have not been examined in cirrhosis-associated hypertrophy.

In cardiac pathological hypertrophy, fibrosis is often found in animal models and in patients with advanced heart failure (8). If hypertrophy happens repeatedly for a month or two, cell death can occur. The dead heart cells are replaced by fibroblasts, and when this happens often enough, fibrosis can develop, causing heart failure.

Our previous data showed that carbon tetra-chloride (CCl₄)-induced cirrhosis can induce pathological cardiac hypertrophy (25). Moreover, we found that two herbal remedies Ocimum gratissimum extract (OGE), found to be rich in antioxidants and possesses many therapeutic functions (2, 3, 5, 6, 9, 11, 17, 26, 27, 35, 36), and silymarin, a standardized extract of the milk thistle (Silibum marianum L. Gaertner) (43), can inhibit liver fibrosis using the CCl₄ model (32) and protect against CCl₄-induced cirrhotic cardiac hypertrophy (25) and cardiomyocyte apoptosis (24). In this study, we want to determine if fibrosis is already present in CCl₄-induced cirrhosis-associated cardiac hypertrophy, as well as find out whether OGE and silymarin can protect against CCl₄-induced cirrhotic cardiac cell fibrosis.

Materials and Methods

Preparation of OGE

Leaves of Ocimum gratissimum were harvested and washed with distilled water followed by homogenization with distilled water using polytron. The homogenate was incubated at 95°C for 1 h and then filtered through two layers of gauze. The filtrate was centrifuged at 20,000 g, 4°C for 15 min to remove insoluble pellets and the supernatant (OGE) was thereafter collected, lyophilized and stored at -20°C until use. The final extract (OGE) was composed of 11.1% polyphenol (including 0.03% catachin, 0.27% caffeic acid, 0.37% epicatechin and 3.27% rutin).

Animals and Treatment

Fourty male Wistar rats weighing 200-240 g were housed in conventional cages with free access to water and rodent chow at 20-22°C with a 12 h light-dark cycle. The rats were divided evenly into five experimental groups. All procedures involving laboratory animal use were in accordance with the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMU) for the care and use of laboratory animals. Rats were treated intraperitoneally with CCl₄ (8% CCl₄/corn oil; 1 ml/kg body weight twice a week; Monday and Thursday) for 8 weeks, as described by Hernandez-Munoz et al. (15) with some modification. At the same time, the rats were treated with various dosages of OGE (0-40 mg/kg body weight), or silymarin orally (200 mg/kg body weight; four times a week; Tuesday, Wednesday, Friday and Saturday) (33, 34). The control rats were treated with corn oil and fed a normal diet. At the end of the experiment, blood and heart were immediately obtained after the animals were sacrificed.

Histological Examinations

The heart was fixed in 10% formalin, processed using routine histology procedures, embedded in paraffin, cut in 5 μm sections and mounted on a slide. The samples were stained with hematoxylin and eosin for histopathological examination. Masson stain was used to confirm CCl₄-induced heart fibrosis evidenced by fiber extension and collagen accumulation.

Preparation of Tissue Extract

All procedures were performed at 4°C. The heart samples were lysed by 30 strokes using a Konetes homogenizer at a ratio of 100 mg tissue/1 ml lysis buffer. The lysis buffer consisted of 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40, and 0.3% sodium deoxycholate. These homogenates were centrifuged at 100,000 g for 1 h at 4°C. The supernatant was stored at -70°C for western blot assay.

Electrophoresis and Western Blot

Tissue extract samples are prepared as described above. Sodiumdoexyl sulfate-polyacrylamide gel electrophoresis is carried out as described by Laemmli
et al. (23) using 10% polyacrylamide gels. After samples are electrophoresed at 140 V for 3.5 h, the gels are equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. Electrophoresed proteins are transferred to nitrocellulose paper (Amersham, Hybond-C Extra supported, 0.45 Micro) using a Hoeger Scientific Instruments Transphor Units at 100 mA for 14 h. The nitrocellulose paper is incubated at room temperature for 2 h in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20 and 3% (v/v) fetal bovine serum. Various antibodies and α-tubulin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA. USA) are diluted to 1:2000 in antibody binding buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20 and 1% (v/v) fetal bovine serum. Incubations are performed at room temperature for 3.5 h. The immunoblots were washed three times in 50 ml blotting buffer for 10 min and then immersed for 1 h in the second antibody solution containing horseradish peroxidase goat anti-rabbit or anti-mouse IgG (Promega) for various antibodies and horseradish peroxidase goat anti-mouse IgG (Promega) for α-tubulin which were diluted in binding buffer to 1000-fold. After washing with blocking buffer, the membrane was visualized using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical Analysis

The experimental results are expressed as the means ± SD. Data were assessed using analysis of variance (ANOVA). Student’s t-test was used in the comparison between groups. A P value less than 0.05 was considered statistically different.

Results

Expression of Cardiac Fibrosis in the Heart of CCl₄ Treated Rats

The sample used here is from the previous experiment (24), where liver cirrhosis and cardiac hypertrophy were observed in CCl₄ treatment in the form of thicker ventricle wall and heavier heart weight. In order to determine that cardiac fibrosis exists in CCl₄ induced cirrhosis associated cardiac hypertrophy, Masson’s trichrome staining, which specifically stains collagen fibers blue, was performed. The extent of the trichrome staining was analyzed by a densitometer (Alphalmager 2000, Alpha Innotech Corporation). The
percent of area of trichrome staining from 8 animals was ~5% in CCl₄-treated-only group, meaning that fibrosis is present (Fig. 1). Furthermore, in the sample co-treated with OGE and silymarin, there is very little trichrome staining, indicating attenuation by OGE or silymarin of CCl₄-induced cardiac fibrosis.

Expression of Cardiac Fibrosis Related Genes in the Heart of CCl₄ Treated Rats

It has been reported that fibrosis can occur through TGF-β activation and then through the Smad pathway (28). We hypothesize that CCl₄ intoxication may induce the activation of TGF-β and its various downstream pathways and performed a Western blotting analysis to confirm this. The data shows that the expression of TGF-β, p-Smad 2/3, and CTGF were significantly increased in CCl₄ induced cardiac damage compared to control (P < 0.05) (Fig. 2), whereas the matrix metalloproteinase expressions (MMP9, MMP2, TIMP1, and TIMP2) exhibited no significant changes (Fig. 3). This is interesting since matrix metalloproteinase is usually reported to have a vital role in fibrosis. In addition, low dose OGE and silymarin treatment significantly inhibited the elevation of the TGF-β signaling pathway expression (P < 0.05) (Fig. 2), and high dose OGE was also able to inhibit their elevation, but not significantly.

Discussion

Cardiac hypertrophy is classified into physiological and pathological hypertrophy (37), the former being a natural bodily response to physical stress such as maturation, pregnancy, and exercise, the latter being a response to organ damage, inflammation, or toxicity. The previous study in which we studied the molecular mechanism of actions in cirrhosis-associated cardiac hypertrophy using the CCl₄-induced liver cirrhosis model (25) found that this type of cardiac damage can result in the increased expression of the IL-6 signaling pathway related genes MEK5, ERK5, JAK, and STAT3 as well as cardiac hypertrophy related markers such as NFAT3, GATA4.
and fetal gene BNP. Since visual cues for pathological hypertrophy (7, 21, 30, 45), such as loss of tissue integrity, were present, the researchers suggest that CCl₄-induced cirrhosis associated cardiac hypertrophy is a pathological response to the liver condition.

It has been reported that pathological hypertrophy is associated with the loss of myocytes, which when occurs is replaced with excessive extracellular matrix (ECM) (31). The deposit of ECM, present in cardiac fibrosis is primarily type-1 collagen in content, which when excessively accumulated can stiffen the ventricles, disable the electrical coupling of cardiac myocytes with extracellular matrix proteins, and reduce capillary density, which lead to the impairment of contraction and relaxation and contribute to the transition from hypertrophy to cardiac failure (13). In our test, trichrome staining confirms that CCl₄ induced liver cirrhosis-associated cardiac hypertrophy is of the pathological kind.

Cardiac fibrosis, the excessive accumulation of ECM (31), can be stimulated by pro-fibrotic cytokines such as transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF). One of the most studied mediators, TGF-β1, is secreted by cardiac fibroblasts in response to stimuli such as Ang II (19) and appears to be mediated in part via Smads and TGF-β-activated kinase-1 (TAK1) in the increased transcription of ECM (19, 38). CTGF has also emerged to be an important mediator of fibrosis in many pathological situations such as the stimulation of collagen synthesis (20, 39, 44). TGF-β can induce CTGF production in cardiac fibroblasts and cardiomyocytes (10), and the CTGF protein can also directly bind TGF-β protein, leading to enhanced TGF-β signaling (1). Our data also shows that the expression of TGF-β, p-Smad 2/3, and CTGF were increased in CCl₄ induced cardiac damage and also demonstrates that their overexpression may be reversed by OGE or silymarin treatment thus lowering liver cirrhosis, which confirms the importance of the growth fac-

![Western blotting analysis](image-url)

Fig. 3. The relative expressions of MMP-9, MMP-2, TIMP-1 and TIMP-2 of the heart of CCl₄ treated rats by Western blotting analysis.

Western blotting analysis (top) and quantitative analysis (bottom). The individual severity rates in rats were expressed as mean ± SE, n = 8.
patients with liver cirrhosis.

In addition to CTGF and TGF-β1, MMP-2, MMP-9 and TIMP-1 were also observed in cardiac fibrosis (4). MMP genes have been known to be induced by cytokines such as TGF-β, and dysregulation of MMPs and TIMPs may contribute to maladaptive left ventricle remodeling and the progression to heart failure (16, 29). The matrix metalloproteinase expressions (MMP-9, MMP-2, TIMP-1, and TIMP-2), however, exhibited no changes in our experiment even though the TGF-β1 signaling pathway is increased and activated. Perhaps other MMPs than the ones tested are involved in the cascade and further experiments are needed in the investigation.

In summary, in previous studies, where we studied the molecular mechanism of actions in cirrhosis-associated cardiac hypertrophy (25) and the progression of cell disintegration and cell apoptosis (pending publication), it was found that this type of cardiac damage can result in pathological hypertrophy (Fig. 4), and the present finding of type-1 collagen in the cardiac tissue and the western blotting analysis confirm that hypertrophy caused by CCl₄ induced cirrhosis associated damage can lead to eventual fibrosis through the TGF-β signaling pathway. Furthermore, OGE and silymarin in the form of herbal supplements can protect cardiac cells from CCl₄ induced damage possibly by inhibiting the activation of the TGF-β signaling pathway and is proposed as a viable option for the protection of cardiac tissue in patients with liver cirrhosis.

Acknowledgments

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