Clinical Nutrition 32 (2013) 252–258

Contents lists available at SciVerse ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

# Garlic extract attenuating rat liver fibrosis by inhibiting TGF-β1

Giuseppe D'Argenio [a,b,*], Giovanna Mazzone [a], Maria T. Ribecco [a], Vincenzo Lembo [a], Paola Vitaglione [c], Maria Guarino [a], Filomena Morisco [a,c], Manuela Napolitano [a], Vincenzo Fogliano [c], Nicola Caporaso [a]

[a] Gastroenterology Unit, Department of Clinical and Experimental Medicine, Federico II University of Naples, Italy
[b] Institute of Protein Biochemistry, CNR, Naples, Italy
[c] Department of Food Science, Federico II University, Naples, Italy

ARTICLE INFO

*Article history:*
Received 16 March 2012
Accepted 9 July 2012

*Keywords:*
Liver
Fibrosis
Garlic
Transglutaminase
TGF-β1

SUMMARY

*Background & aims:* We previously demonstrated the efficacy of garlic extract (GE) in the prevention of rat liver fibrosis by inhibiting tissue transglutaminase (tTG) activity. In the present study we aimed to evaluate the potential of GE in the regression of liver fibrosis and the underlining mechanism.
*Methods:* Male Wistar rats were i.p. injected, twice a week, for 7 weeks, with $CCl_4$ to develop liver fibrosis. Successively, a group was immediately sacrificed, while the remaining two groups received the GE or the vehicle, respectively, over the following 2 wks. A group of normal rats was also included in the study. Liver function, histology, and collagen deposition in parallel with gene and protein expression of α-SMA, tTG, TGF-β1, SEMA-7A, and metalloproteinase inhibitor 1 (TIMP1) as well as measure of active by total TGF-β1 were assessed.
*Results:* $CCl_4$ administration increased alanine-aminotransferase (ALT) activity, hepatic collagen deposition and gene and protein expression of all monitored markers. GE, but not the sole vehicle, restored liver histology and function by decreasing fibrogenesis markers (α-SMA, tTG, TGF-β1, SEMA-7A and TIMP1). Active by total TGF-β1 was significantly reduced ($p < 0.05$) in GE treated rats compared to the $CCl_4$ at 7 weeks, and vehicle rats.
*Conclusions:* These findings concurrently suggested that GE elicited therapeutic effect against liver fibrosis. Regression of liver fibrosis occurred by reducing myofibroblasts (through modulation of HSCs activation mechanisms), remodelling extracellular matrix (through increase of its degradation) and regenerating liver tissue and functions: three processes regulated by fine mechanisms where active TGF-β1 and tTG play a central role.


## 1. Introduction

Liver fibrosis is a worldwide health problem because it gradually evolves into cirrhosis, that is associated with high morbidity and mortality.[1] Thus the need to develop antifibrotic treatments that can prevent, halt, or even reverse liver fibrosis or cirrhosis.[2] Since liver fibrosis results from a dynamic process, characterized by a preponderance of fibrogenesis (the excess synthesis and deposition of extracellular matrix, ECM), over its removal (fibrolysis), even advanced fibrosis and possibly cirrhosis can regress once the fibrogenic trigger is eliminated and fibrolysis prevails over fibrogenesis.[3,4]

As a consequence all the cells, signalling pathways, and molecules critical for fibrosis progression or reversal may be also the targets for antifibrotic therapies.[2] In this framework particular attention has to be paid to 1) the profibrogenic growth factors, cytokines, mediators and cells upstream hepatic stellate cells (HSCs) activation and recruitment, 2) the intracellular pathways in both HSCs and in cells upstream of their activation and 3) the stimulation of fibrolytic processes to reverse existing fibrosis.[2]

Evidence indicates that activated HSCs are central to the process of fibrosis and its reversal.[5] They are myofibroblasts consisting in a heterogeneous cell population arising mainly from transdifferentiation of quiescent HSCs, and liver fibroblasts, but also (to a lesser extent) from activated/injured liver epithelia and from bone-marrow-derived circulating fibrocytes.[6–8] Active HSCs are characterized by increased proliferation, migration, and contractility, and a relative resistance to apoptosis.[9,10] At molecular level they show increased expression of α-smooth muscle actin (α-SMA) and procollagen-I that parallels the great ability of the activated

* Corresponding author. Dipartimento di Medicina Clinica e Sperimentale, Gastroenterologia, Facoltà di Medicina, Università Federico II, Via Pansini 5, 80131 Napoli, Italy. Tel.: +39 81 7462706.
*E-mail address:* dargenio@unina.it (G. D'Argenio).

0261-5614/$ – see front matter 
http://dx.doi.org/10.1016/j.clnu.2012.07.001

HSCs to depose collagens and other matrix proteins in the extracellular space.[11] The increased collagen synthesis that accompanies fibrosis progression is also associated to collagen accumulation. In fact activated HSCs present an altered regulation of matrix remodelling enzymes, such as metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), modulating matrix degradation and production, respectively.[12] The direct and unique participation of tissue transglutaminase (tTG) (over-expressed during fibrogenesis), to stabilization and reversion of collagen fibres in ECM, via forming stable and proteolytic resistant ε(γ-glutamyl)-lysine dipeptide cross-links, is actually controversial as recent evidence suggests that several mechanisms coexist *in vivo*[13–15] while the indirect role of tTG via activation of TGF-β1 has been recently ascertained.[16] This pleiotropic cytokine is secreted in the latent form and, when it is activated, it plays a central role in fibrogenesis. In fact, active TGF-β1 induces the activation of HSCs[9,17,18] and modulates the expression and secretion of a number of proteases and their regulators including plasmin, plasminogen activators, and plasminogen activators inhibitors-1 (PAI-1), MMPs and TIMPs.[19] Recent studies have demonstrated that TGF-β1 is also a potent stimulator of the membrane-bound protein Semaphorin 7A (SEMA-7A), acting in the pathogenesis of pulmonary fibrosis.[20,21] Most importantly TGF-β1 can auto-induce its own production thus subsequently amplifying its actions.[22]

Several studies have been conducted to find natural products able to prevent or retard liver fibrosis progression. We have recently demonstrated that a garlic extract (GE), can reduce liver fibrosis progression,[23] probably through its organosulphur compounds having a chemical structure very similar to Cystamine (β-mercaptoethanolamine disulphide), the well known chemical inhibitor of tTG.[24,25] In addition, it has recently been suggested[15] that liver fibrosis may persist for a long time even after successful pharmacological treatment of hepatitis, therefore a fibrolytic therapy to rapidly reverse advanced fibrosis/cirrhosis has been claimed.

In this view we aimed to evaluate the effect of GE on liver fibrosis attenuation in rats. To do this, liver fibrosis was chemically induced and, upon suspension of the liver insult, rats were treated with GE for the successive 2 weeks. Histology and different markers of fibrogenesis and fibrolysis on liver tissue were evaluated.

## 2. Materials and methods

### 2.1. Garlic extract preparation and characterization

Garlic extract was prepared and characterized as previously described.[23] Briefly: peeled garlic (500 g) was freeze dried and reduced to a fine powder in an electric mixer. The powder was extracted overnight in ethanol-water: 50:50 (v/v). The GE was then evaporated under vacuum at room temperature until its volume was reduced to one half. Successively the extract was freeze dried, reduced to a fine powder by a mixer and stored at 4 °C until the use. This procedure gave an extraction yield of 3.4%. Identification and quantitative determination of the organosulphur compounds present in GE was carried out by HPLC/MS/MS.

### 2.2. Study design

Twenty male Wistar albino rats (Harlan Laboratories, Udine Italy) weighting 200–250 g were included in the study. Rats were housed in a room at a mean constant temperature of 22 °C with a 12-h light–dark cycle and had free access to standard pellet chow and water.

Rats were randomly divided into 4 groups of 5 rats each. In 15 rats liver fibrosis was induced by intraperitoneal (i.p.) injections of carbon tetrachloride ($CCl_4$) 0.2 mL/100 g b.w., twice a week for 7 weeks according to a well-established model.[15] Five rats were sacrificed immediately after the induction of liver fibrosis (7 weeks), while the remaining 2 group of rats were sacrificed 2 weeks later after having received daily by i.p. injection the GE (200 mg/kg b.w.) or the vehicle (i.e. water), respectively. The 5 rats who were not injected with $CCl_4$ were the healthy controls and were sacrificed at 7th week. The dose of GE was decided on the basis of our previous study.[23] This concentration of GE inhibits more than 80% of TG activity when incubated in vitro with liver homogenate used as enzyme source. The optimal timing of sacrifice was established on the basis of our previous experience, and on data from recent articles[14,15] showing that the spontaneous recovery of liver injury is a very slow process that certainly is not completed within 2 weeks.

At the end of treatment, rats were sacrificed, and blood and liver biopsies were collected for histological and biochemical evaluations. The remaining liver tissues were snap-frozen to extract total RNA and proteins.

The study was approved by the Federico II University School of Veterinary Medicine Animal Care.

### 2.3. Histology

Samples from liver tissue were fixed, dehydrated in graded ethanol, and embedded in paraffin. Sections (4 μm) were processed for haematoxylin and eosin staining and slides were observed using a blinded protocol. Tissue structure in the liver was analyzed in samples from each group. Fibrosis was graded as previously described[26]: grade 0: no damage; grade 1: increase in collagen without septa formation; grade 2: formation of incomplete septa; grade 3: complete but thin septa; and grade 4: presence of thick septa. For the detection of collagen content, sections were stained with picrosirius red solution; for the quantification of collagen deposition, a videomicroscope (Sony) equipped with motor stage and the Quantimed 500MC (Leica, Germany) software were used.

### 2.4. ELISA for determination of the active TGF-β1

The hepatic concentration of TGF-β1 was assessed by ELISA (Quantikine TGF-β1 ELISA kit, R&D Systems, USA) following the manufacturer's instructions. Liver tissues were homogenized with 10 μL/mg extraction buffer (50 mmol/L HEPES, 50 mmol/L tetrasodium pyrophosphate, 100 mmol/L sodium fluoride, 10 ml EDTA pH 7.4, 10 mmol/L Na-orthovanadate, and protease inhibitor cocktail). The concentration of active TGF-β1 was obtained from samples that were not acidified before developing the assay. Active TGF-β was expressed as percentage of total TGF-β.

### 2.5. Serum biochemical parameters

To assess liver function, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin levels were determined by using a Modular Auto analyzer (Roche Diagnostics, GmbH, D-68298, Mannheim, Germany).

### 2.6. Western blotting

Liver samples, about 20–30 mg, were homogenised in 1500 μl of RIPA lysis buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma, St Louis, Missouri, USA), 0.5 mM dithiothreitol and 0.5% phenylmethylsulphonyl fluoride. Liver homogenates were run on 10% SDS/polyacrylamide gel according to Laemmli.[27] Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Biorad transblot apparatus) and detected using