Effect of Gallic Acid on Regression of Murine CCL4-Induced Hepatic Cirrhosis

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Abstract: Hepatic cirrhosis can be induced by chronic exposure to reactive oxygen species (ROS). However, phenolic compounds, such as gallic acid (GA), appear to inhibit ROS-induced oxidative stress. We aimed to investigate the effects of GA on murine hepatic fibrogenesis. The effects of GA were evaluated on the regression of liver cirrhosis that was induced by chronic intraperitoneal CCL4 administration (20% v/v) in C57 mice for a period of 10 weeks. The animals were treated intraperitoneally and distributed in three groups, as follows: SHAM group – 8 weeks with Olive oil (CCL4 solution vehicle); C group – 8 weeks with CCL4 solution, and then 2 more weeks with deionized water (Gallic Acid solution vehicle); and C + GA group – 8 weeks with CCL4 solution, and then 2 more weeks with Gallic Acid solution (100 mg/Kg/day) on alternated days. The cirrhosis and inflammation-related factors were estimated using histological, western blotting and PCR-RT analysis. There was a significant decrease in the collagen deposition, as indicated by Sirius red staining, in the C + GA group compared to the C group (p<0.05). This improvement was accompanied by a reduction in the number of α-SMA-positive cells observed in these animals (p<0.05). The expression of the Procollagen α1(I), TGFβ1 and TIMP1 genes was also diminished by GA administration compared to the control (p<0.001). Additionally, proteomic studies revealed reduced p65 NFκB and p38 MAPK protein levels in the C + GA group. These findings reveal the effect of GA on the regression of cirrhosis. The mechanisms of this process might involve the anti-inflammatory activity of GA, which represses the TGFβ1, p65 NFκB, and p38 MAPK-mediated signaling pathways.

Keywords: Phenolic Compounds, Gallic Acid, Liver Cirrhosis, Oxidative Stress, Reactive Oxygen Species, Hepatic Stellate Cells

1. Introduction

Hepatic cirrhosis represents a common cause of morbidity/mortality in the Occidental world [1]. The pathogenesis of hepatic cirrhosis during chronic liver disease involves the proliferation and activation of hepatic stellate cells (HSCs), which are associated with the deposition of collagen and other extracellular matrix (ECM) components [2]. The activation of HSCs also results in the increased expression of alpha smooth muscle actin (α-SMA) [3]. In addition, the activated HSCs stimulate the inhibitor of matrix metalloproteinase 1 (TIMP1), which blocks the matrix metalloproteinase 1 (MMP1)-mediated degradation of the ECM, consequently augmenting collagen levels [4, 5].

Several mechanisms have been implicated in the process of HSC stimulation, but oxidative stress has been described with increasing importance [6]. Some authors have suggested that oxidative stress products - mainly reactive oxygen species (ROS) and their deleterious effects - might represent a link between chronic injury and liver fibrosis [7, 8]. Additionally, lipid peroxidation products can induce the release of collagen by cultivated HSCs [9, 10]. The deficiency of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, one of the most important ROS production-related enzymes, has been associated with...
restricted fibrosis in carbon tetrachloride-treated mice [11]. Moreover, the inhibition of pro-oxidative stress-derived factors, such as nuclear factor kappa B (NFkB), might reduce hepatic fibrogenesis [12]. The levels of some cytokines, such as palate derived growth factor (PDGF) and transforming growth factor beta-1 (TGF\(\beta\)1), are augmented in response to the ROS and can stimulate the activation of HSCs. PDGF has been associated with the increased production of hydrogen peroxide, a key type of ROS that is produced during the progression of human hepatic fibrosis. In contrast, during liver fibrogenesis, TGF\(\beta\)1 reduces the levels of glutathione, which is an important endogenous antioxidant [7, 13, 14]. Therefore, antioxidants might represent potential therapeutic agents for the prevention of hepatic fibrogenesis and the regression of cirrhosis [15].

Recently, a new and powerful class of antioxidants has been identified, the (poly) phenolic compounds. The (poly) phenols are abundant plant constituents that are consumed in a regular diet. The most significant effect of the (poly) phenolic compounds, in addition to neutralizing free radicals, is the inactivation of catalytic metals via the chelating and reduction of superoxide ions to more stable hydroxyl derivatives and via synergistic interactions with other reducing compounds [14].

Gallic Acid (GA) is a (poly) phenolic compound that is found in various plant species [16]. Several therapeutic effects have been correlated with GA, predominantly for its anti-inflammatory and antioxidant activities [16, 17]. Although GA has been described to elicit a protective role on hepatocytes [18], other studies have suggested that GA can prevent the production of ROS and the hyperproduction of collagen during cardiac fibrosis [19] and in the in vitro inhibition of fibroblast proliferation and contractility [20]. Also, GA has been found to elicit cytotoxicity to a diverse range of tumor cells and renal fibroblasts [20, 21]. In hepatic fibrosis, the GA showed prophylactic effect by inhibiting the fibrogenic activity of CCL\(_4\) [22]. Although GA can potentially act as an anti-fibrogenic factor in others tissue and liver, the effects of GA in regression of hepatic fibrosis remain to be determined. Thus, we aimed to investigate the effects of GA in regression of murine hepatic cirrhosis.

2. Method and Material

2.1. Animals

C57 mice (n=46), weighing 20-25 g were housed under standard conditions, including temperature and a 12:12-h light/dark cycle, with food and water provided ad libitum. Animal management conformed to the International Guiding Principles for Biomedical Research Involving Animals and was approved by our institutional animal ethics committee (n°.174/2010).

2.2. Experimental Design

Animals were randomly divided into the following three distinct groups:

- **Group 1 - SHAM (n=6):** the animals were inoculated intraperitoneally (i.p.) with olive oil (CCL\(_4\) solution vehicle) twice weekly for a period of 8 weeks.
- **Group 2 – Control group (n=10):** the animals were inoculated with a CCL\(_4\) solution of 1 ml/kg/olive oil (20% v/v) i.p. twice weekly for a period of 8 weeks [23]. Subsequently, the animals received deionized water i.p. on alternate days for an additional period of 2 weeks.
- **Group 3 – Regression group (n=10):** the animals were inoculated with the CCL\(_4\) solution 1 ml/kg/olive oil (20% v/v) i.p. twice weekly for 8 weeks [23]. Subsequently, they received GA (100 mg/kg/day) [18] i.p. on alternate days for an additional period of 2 weeks.

Subsequently, the animals were sacrificed under anesthesia with intramuscular ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight). Fragments of liver tissue were fixed in 10% buffered formalin for 48h and embedded in paraffin. Small liver samples were also collected in RNA stabilizing solution (RNALater RNA Stabilization Reagent, Sigma-Aldrich, St. Louis, MO, USA), frozen in liquid nitrogen and stored at -80°C.

2.3. Histological Analysis

Liver samples were fixed in 10% buffered formalin for 24 hours, embedded in paraffin and subjected to Sirius Red staining. Collagen deposition was measured as the percentage of Sirius Red-positive staining (red) cells in ten random fields (20X microscopic magnification) using Image J software (Image J, 1.33u, NIH, USA).

The liver preparations were also submitted for immunohistochemical analysis for alpha smooth muscle actin (\(\alpha\)-SMA, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution) using an avidin-biotin peroxidase system (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc., Burlingame, CA, USA). The immunolabeling was detected using the NovaRed kit (Vector Laboratories Inc.) and counterstained with Harris’s hematoxylin. The \(\alpha\)-SMA-positive cells were quantified randomly in at least 30 representative high-power fields (40X). The results are expressed as the number of \(\alpha\)-SMA-positive cells per microscopic field.

2.4. Gene Expression by Real Time PCR

The tissue samples were subjected to total RNA extraction using a RNeasy mini Kit (QIAGEN, Hilden Germany). Complementary DNA was obtained via the reverse transcription of 5 mg of total RNA using a reverse transcription kit (Ready-To-Go You-Prime First-Strand Beads and pd) (N)6 Random Hexamers, Amersham Biosciences Corp., Piscataway, NJ, USA). The simultaneous quantification of gene amplification was performed using a StepOnePlus™ kit (Applied Biosystems, Foster City, CA, USA) with primers specific for the Procollagen \(\alpha\)(I), TGF\(\beta\)1, TIMP1 and 18S (Assays-on-Demand Gene Expression Products, Applied Biosystems) genes and Taq polymerase (TaqMan Universal PCR Master Mix, No
AmpErase UNG - 2X, Applied Biosystems). The ΔΔCT method was used to analyze the difference between samples, and the 18S gene was used as a reference.

### 2.5. Protein Quantification by Western Blotting

The protein levels of p38 MAPK and p65 NFκB were quantified by Western blotting. Cell extracts were obtained from frozen liver samples using a Triton® X100-based lysis buffer containing protease and phosphatase inhibitors. The protein samples (100 mg) were resolved on 10% SDS-acrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against p38 MAPK and p65 NFkB (Santa Cruz Biotechnology, 1:1000 dilution), and β-actin (Sigma-Aldrich, 1:1000 dilution) as a loading control. Next, the membranes were washed with buffer and incubated with a peroxidase-conjugated secondary antibody. The proteins were detected by electrochemiluminescence (ECL, Amersham Biosciences Corp.). The resulting blots were scanned using an ImageReader LAS-3000 imaging densitometer (Fujifilm, Tokyo, Japan), and the optical densities of the specific protein bands were quantified using the ImageGauge software (Fujifilm).

### 2.6. Statistical Analysis

Given the nature of the data, the nonparametric Kruskal-Wallis and Mann-Whitney U tests were applied for statistical comparisons. Probability values less than 0.05 were considered statistically significant. The statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA).

### 3. Results

#### 3.1. Histological Analysis

The photomicrographs show the collagen stained by Sirius red in the C and C + GA groups (Fig.1A and 1B, respectively). It may be noted the reduction of the fibrotic state in the C + GA group (Fig. 1B), which only showed a fibrous septum (FS), when compared to the C group (Fig. 1A), which presented a regenerative nodule (RN). Our data revealed a significant increase in collagen deposits in all of the animals exposed to CCl₄ compared to the SHAM group (0.46 ± 0.18) (p<0.01). Treatment with GA decreased collagen levels in the C + GA group (1.49 ± 0.19) in contrast to the C group (2.17 ± 0.6) (p<0.05) (Fig. 1C).

Consistently, we observed fewer α-SMA-positive cells in animals treated with GA in the C + GA group (Fig. 2B) compared to the C group (Fig. 2A). Moreover, the α-SMA-positive cells were more frequently observed in the CCl₄-treated group compared to the SHAM group (0.45 ± 0.36) (p<0.001). The number of α-SMA-positive cells was reduced in the C + GA group (11.45 ± 3.35) compared to the C group (18.17 ± 4.76) (p<0.001) (Fig. 2C).

#### 3.2. Gene Expression Evaluation

The graphics display the quantification of Procollagen α1(I) (Fig. 3A), TGFβ1 (Fig. 3B) and TIMP1 (Fig. 3C) expression in the C + GA and C groups. The expression of Procollagen α1(I) (1.89 ± 0.68), TGFβ1 (1.11 ± 0.49) and TIMP1 (0.92 ± 0.43) was significantly reduced in the C + GA group in relation to the C group (7.65 ± 0.71; 6.17 ± 0.69 and 5.20 ± 0.67) (p<0.001; p<0.001; p<0.001, respectively).

#### 3.3. Protein Quantification

The p38 MAPK protein levels were similar in the C + GA (+0.4-fold) and C (+0.6-fold) groups. Considering the p65 NFκB levels, the C + GA group exhibited a significant reduction in p65 NFκB protein levels (+0.3-fold) compared to the C group (+0.7-fold) (Fig. 3D).
4. Discussion

The findings of this study revealed the anti-fibrogenic effect of GA in established cases of cirrhosis. Initially, the major consequences and implicated mechanisms involve activated HSCs. Indeed, during their activation, HSCs overexpress the α-SMA protein, a phenomenon that is correlated with increased hepatic fibrogenesis [5]. In this study, we observed a reduction of α-SMA-positive cells in the C + GA group.

An important outcome of HSC activation is the increased expression of Procollagen α1 [9] and the consequent collagen protein deposition [24]. Treatment with GA resulted in apparent anti-fibrogenic activity, which suppressed the expression of Procollagen α1 [9] and the collagen labeling by Sirius red. These findings suggest a significant effect of GA with a significant role in the regression of cirrhosis.

Moreover, in addition to the TIMP1-mediated inhibition of MMP1 activity and consequent ECM collagen accumulation, TIMP1 also exhibits an anti-apoptotic role in HSCs, which augments the activation of the HSC population and fibrosis [5, 25]. Consistently, we observed decreased TIMP1 expression in the animals that were treated with GA. This reduction corroborates results founded in other studies [11] and was also accompanied by a reduced number of α-SMA-positive cells and collagen deposits.

Apart from the mechanisms that directly involve collagen deposition, factors related to HSC activation should be considered. A classic factor that stimulates HSC activation and consequent fibrogenesis is TGFβ1 [4]. We also observed that GA treatment reduced the expression of TGFβ1, which was accompanied by a decrease of α-SMA-positive cells and collagen deposits. Another important pathway comprises transcription factors such as NFκB. The activation of NFκB promotes the transcription of diverse proteins, particularly TGFβ1 [26]. In this study, a significant reduction of activated NFκB (p65 NFκB) was observed in the C + AG group. Because p65 NFκB is considered a pro-inflammatory transcription factor that mediates cellular response to oxidative stress [4, 27, 28], these results suggest that GA might elicit a potential anti-inflammatory effect to the CCl4 exposure.

Another mechanism involved in hepatic fibrosis, which is secondary to oxidative stress, is mediated by mitogen-activated protein kinases (MAPK). p38 MAPK is a member of the MAPK family that can be activated by TGFβ1 and that can be considered an additional factor that stimulates the activation and proliferation of HSCs [5]. Therefore, increased p38 MAPK protein is associated with increased hepatic fibrosis development [24]. We observed that the GA treatments reduced the p38 MAPK protein in the animals with established cirrhosis.

5. Conclusions

These findings reveals that the GA have an important role in the regression of hepatic cirrhosis and suggests that the predominant mechanisms of its effects involves the repression of the anti-apoptotic activity of p65 NFκB in the HSC and the reduction of TGFβ1 and consequently the reduction of the p38 MAPK in established cases of cirrhosis.

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References


