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Liver fibrosis in mice induced by carbon tetrachloride and its reversion by luteolin

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ABSTRACT

Hepatic fibrosis is effusive wound healing process in which excessive connective tissue builds up in the liver. Because specific treatments to stop progressive fibrosis of the liver are not available, we have investigated the effects of luteolin on carbon tetrachloride (CCl_4)-induced hepatic fibrosis. Male Balb/C mice were treated with CCl_4 (0.4 ml/kg) intraperitoneally (i.p.), twice a week for 6 weeks. Luteolin was administered i.p. once daily for next 2 weeks, in doses of 10, 25, and 50 mg/kg of body weight. The CCl_4 control group has been observed for spontaneous reversion of fibrosis. CCl_4-intoxication increased serum aminotransferase and alkaline phosphatase levels and disturbed hepatic antioxidative status. Most of these parameters were spontaneously normalized in the CCl_4 control group, although the progression of liver fibrosis was observed histologically. Luteolin treatment has increased hepatic matrix metalloproteinase-9 levels and metallothionein (MT) I/II expression, eliminated fibrinous deposits and restored architecture of the liver in a dose-dependent manner. Concomitantly, the expression of glial fibrillary acidic protein and α-smooth muscle actin indicated deactivation of hepatic stellate cells. Our results suggest the therapeutic effects of luteolin on CCl_4-induced liver fibrosis by promoting extracellular matrix degradation in the fibrotic liver tissue and the strong enhancement of hepatic regenerative capability, with MTs as a critical mediator of liver regeneration.

Introduction

Liver fibrosis is a frequent event which follows a repeated or chronic insult of sufficient intensity to trigger a "wound healing"-like reaction, characterized by excessive connective tissue deposition in extracellular matrix (ECM). Chronic carbon tetrachloride (CCl_4) intoxication is a well-known model for producing oxidative stress and chemical hepatic injury. Its biotransformation produces hepatotoxic metabolites, the highly reactive trichloromethyl free radical, which are further converted to the peroxytrichloromethyl radical (Williams and Burk, 1990). Reactive oxidant species likely contribute to both onset and progression of fibrosis (Poli, 2000). Antioxidant treatment *in vivo* seems to be effective in preventing or reducing chronic liver damage and fibrosis (Parola and Robino, 2001). Polyphenols, naturally occurring antioxidants in fruits, vegetables, and plant-derived beverages such as tea and wine, have been associated with a variety of beneficial properties (Havsteen, 2002). The flavone luteolin (3′,4′,5,7-tetrahydroxyflavone) is an important member of the flavonoid family, present in glycosylated forms and as aglycone in various plants (Shimoi et al., 1998). Luteolin is reported to have anti-inflammatory (Ziyan et al., 2007; Veda et al., 2002), antioxidant (Perez-Garcia et al., 2000), antiallergic (Veda et al., 2002), antitumorigenic (Ju et al., 2007), anxiolytic-like (Coleta et al., 2008), and vasorelaxative properties (Woodman and Chan, 2004). Previously, we have shown a hepatoprotective activity of luteolin in acute liver damage in mice (Domitrović et al., 2008a, Domitrović et al., 2009).

Hepatic stellate cells (HSCs) are a minor cell type most commonly found in the space of Disse, intercalated between hepatocytes and cells lining the sinusoid, projecting their dendritic processes to nearby hepatocytes and endothelial cells (Blouin et al., 1977, Mermelstein et al., 2001). Upon liver injury, HSCs become activated, converting into myofibroblast-like cells. Activated HSCs proliferate and produce extracellular matrix (ECM), playing a major role in hepatic fibrosis and regeneration (Friedman, 2000). ECM, which consists of collagens and other matrix components such as proteoglycans, fibronectins, and hyaluronic acid (Arthur, 1994), is regulated by a balance of synthesis and enzymatic degradation of ECM. The key enzymes responsible for degradation of all the protein components of ECM and basement membrane are matrix metalloproteinases (MMPs), a zinc-dependent family of endopeptidases. Previous studies have demonstrated that the activity of these enzymes is altered during the processes of fibrogenesis and fibrinolysis (Knittel et al., 2000).

The metallothioneins (MTs), small cysteine-rich heavy metal-binding proteins, participate in an array of protective stress responses. In mice, among the four known MT genes, the MT I and MT II genes are

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most widely expressed. Transcription of these genes is rapidly and dramatically up-regulated in response to agents which cause oxidative stress and/or inflammation (Andrews, 2000). The induction of MT synthesis can protect animals from hepatotoxicity induced by various toxins including CCl_4, but also play a role in repair and regeneration of injured liver (Cherian and Kang, 2006).

Because the specific treatments to stop progressive fibrosis of the liver are not available, the objective of the present study was to investigate the therapeutic effect and mechanisms of action of luteolin in chemically induced liver fibrosis in mice.

Materials and methods

Materials. Luteolin, carbon tetrachloride (CCl_4), olive oil, dimethyl sulfoxide (DMSO), nitric acid (HNO_3), hydrogen peroxide (H_2O_2), and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of the highest grade commercially available.

Animals. Male Balb/c mice from our breeding colony, 2–3 months old, were divided into 6 groups with 5 animals per group. Mice were fed a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% ash, and 2.6% by weight of lipids (native soya oil) to prevent essential fatty acid deficiency. Total energy of the diet was 16.4 MJ/kg. The animals were maintained at 12 h light/dark cycle, at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). All experimental procedures were approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

Experimental design. Mice were given CCl_4 intraperitoneally (i.p.) at a dose of 0.4 ml/kg, dissolved in olive oil, twice a week for 6 weeks (the CCl_4 group), except the control group which received vehicle only. Seventy-two hours after the last CCl_4 injection, the CCl_4 group was killed. The CCl_4 control group was observed for spontaneous resolution of hepatic fibrosis for next 2 weeks. In the luteolin-treated groups, the polyphenolic compound was administered i.p. at a dose of 10, 25, or 50 mg/kg daily for 2 weeks, respectively. These doses were selected on the basis of preliminary studies (Domitrović et al., 2008a, Domitrović et al., 2009). Luteolin was dissolved in DMSO and diluted in saline to the final concentrations. The final concentration of DMSO was less than 1%. Mice from the control and CCl_4 control groups received diluted DMSO solution daily for 2 weeks. Animals were terminated 24 h after the last dose of luteolin or diluted solvent by cervical dislocation. The blood was taken from orbital sinus of ether anesthetized mice. The abdomen of terminated animal was cut open quickly and the liver perfused with isotonic saline, excised, blotted dry, weighed, and divided into samples. The samples were used to assess biochemical parameters, and another was preserved in a 4% phosphate-buffered formalin solution to obtain histological sections.

Hepatotoxicity study. Serum levels of ALT, AST, and ALP as markers of hepatic function, were measured by using a Bio-Tek EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) according to the manufacturer's instructions.

Determination of Cu/Zn SOD activity and GSH concentration. Cu/Zn SOD activity and total GSH, indicators of oxidative stress, were measured spectrophotometrically, using Superoxide Dismutase Assay Kit and Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Protein content in supernatants was estimated by Bradford's method, with bovine serum albumin used as a standard (Bradford, 1976).

Determination of hepatic hydroxyproline. The tissue samples (50 mg) were hydrolyzed in 4 ml 6 M HCl at 110 °C for 24 h. After being filtered through a 0.45-μm filter, 2 ml of samples was extracted and analyzed according to the procedure of Bergman and Loxley (1963). Briefly, sample neutralization was obtained with 10 M NaOH and 3 M HCl. After neutralization, subsequent steps were made in duplicate for each sample. To a 200 μl of the above solution, 400 μl of isopropanol in citrate-acetate-buffered Chloramine T was added. After 4 min, 2.5 ml of Ehrlich reagent was added. Tubes were wrapped in aluminum foil and incubated for 25 min in a water-bath at 60 °C, cooling each sample in tap water, and measuring the absorbance of each sample spectrophotometrically at 550 nm (Cary 100, Varian, Mulgrave, Australia).

Determination of trace elements. Hepatic zinc (Zn) and copper (Cu) content were determined by ion coupled plasma spectrometry (ICPS) using Prodigy ICP Spectrometer (Leeman Labs, Hudson, NH, USA), according to the method previously described (Domitrović et al., 2008a).

Determination of retinol. The hepatic levels of retinol were analyzed by high-performance liquid chromatography (HPLC) according to Hosotani and Kitagawa (2003), as described previously (Domitrović et al., 2008b).

Histopathology. Liver specimens were fixed in 4% phosphate-buffered formalin, embedded in paraffin, and cut into 4 μm thick sections. Sections for histopathological examination were stained with hematoxylin and eosin (H&E) and Mallory trichrome stain using standard procedure.

Immunohistochemical determination of GFAP, α-SMA, and MT I/II. Immunohistochemical studies were performed on paraffin embedded liver tissues using mouse monoclonal anti-MT I+II antibody diluted 1:50 (clone E9; DakoCytomation, Carpinteria, CA, USA), mouse monoclonal anti-GFAP antibody diluted 1:100 (clone 1B4; BD Pharmingen, San Diego, CA, USA), and mouse monoclonal antibody to α-SMA diluted 1:100 (SPM332; Abcam, Cambridge, UK), employing DAKO EnVision+ System, Peroxidase/DAB kit according to the manufacturer's instructions (DAKO Corporation, Carpinteria, CA, USA). Briefly, slides were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, monoclonal antibodies diluted in phosphate-buffered saline supplemented with bovine serum albumin were added to tissue samples and incubated overnight at 4 °C in a humid environment, followed by incubation with peroxidase labeled polymer conjugated to secondary antibodies containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction product was visualized by adding substrate-chromogen diaminobenzidine (DAB) solution, resulting with brownish coloration at antigen sites. Tissues were counterstained with hematoxylin, dehydrated in gradient of alcohol, and mounted with mounting medium. The intensity of staining was graded as weak, moderate, and intense. The specificity of the reaction was confirmed by substitution of primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX51, Tokyo, Japan).

MMP zymography. MMP-2 and MMP-9 activities were analyzed by gelatin zymography assays as described (Kuo et al., 2003), with modifications. After tissue homogenization in radioimmunoprecipitation assay buffer (4 ml of buffer per gram of tissue) containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 1 mM sodium orthovanadate, and 2 μg/ml of each aprotinin, leupeptin and pepstatin. 10 μg of liver tissue protein lysates were separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin, at 4 °C and 150 V for 4 h. Gels were washed for 30 min in 2.5% Triton X-100 to remove the SDS, and briefly