Original Article

Effect of Curcumin on Liver Fibrosis Formation in Rats with Chronic Inflammation Following Diabetics

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Objective: To determine the effects of curcumin on the semiquantitative changes of inflammatory regulators; interleukin (IL)-13, tumor necrosis factor (TNF)-alpha, and ED1 as well as the type I and IV collagen levels in the liver of diabetic rats.

Materials and Methods: Diabetic male rats were induced by streptozotocin intravenous injection (60 mg/kg BW). Rats were divided into three groups; control (C) group, diabetes (DM) group, and diabetes supplemented with curcumin (200 mg/kg BW) (DMC) group. After 12 weeks of curcumin supplementation, the liver tissues were collected. The levels of IL-13, TNF-alpha, type I collagen, and type IV collagen were analyzed by western blot analysis, while the level of ED1, a marker of hepatic macrophages, was analyzed by immunohistochemical method.

Results: The levels of IL-13, TNF-alpha, type I collagen, and type IV collagen were markedly elevated in the DM group compared to the control group. In contrast, the levels of these entire proteins were decreased significantly in the DMC group. In addition, the level of ED1-immunoreactivity significantly decreased in DMC group compared to that in the DM group.

Conclusion: The present results support the hypothesis that curcumin can reduce hepatic inflammation and fibrosis in diabetic liver tissues. Therefore, dietary curcumin might have efficacy to ameliorate diabetic-induced hepatic injury in terms of anti-inflammation and antifibrotic properties.

Keywords: Curcumin, Diabetes, Hepatic injury, Inflammation, TNF-alpha, Fibrosis

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Diabetes mellitus (DM) is one of the chronic metabolic disorders, which leads to elevation of blood glucose concentrations also called hyperglycemia. DM can interfere all body systems, thereby leading to more severe pathological condition, for instance, cardiovascular diseases, nephropathy, neuropathy, and hepatopathy[1]. Previous study has indicated that DM is involved in numerous liver diseases, such as fibrosis, cirrhosis, and hepatocellular carcinoma[2]. Hyperglycemia is the principal cause of diabetic complications, including liver disease. It can accelerate formation of advanced glycation end-products (AGEs) that are generated through a non-enzymatic reaction between reducing sugars and free amino groups of proteins, nucleic acids, or lipids. The excessively high levels of AGEs induce the production of reactive oxygen species (ROS), which can cause oxidative stress[3], resulting in inflammation and cell death. Therefore, the possible mechanism of DM that contributes to hepatic injury might be the combination of increased oxidative stress and attenuation of the inflammatory response.

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AGEs-induced inflammatory responses are via interaction with the receptors located on the cell surface which subsequently activate different signaling pathways and produce inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-6, and IL-18[4]. In addition, IL-13 is a T-helper 2 (Th2) cytokine that is a mediator of allergic inflammation and disease. Previous study has shown that IL-13 plays an important role in the regulation of hepatic glucose production[5]. Moreover, IL-13 also has anti-inflammatory property that is associated with the regulation of the secretion of IL-1beta and TNF-alpha by monocytes. TNF-alpha can trigger multiple signaling pathways associated with inflammation and apoptosis[6]. Previous studies have suggested that macrophages in the liver, also known as Kupffer cells, have a critical role in the pathogenesis of acute and chronic liver diseases, in which they orchestrate inflammation[2,6]. The Kupffer cells and exudate macrophages strongly express ED1 antigen. Therefore, ED1 is being used widely as a marker for exudate macrophages, Kupffer cells, as well as peripheral blood monocytes in rat tissues[7].

Furthermore, chronic hepatic inflammation has been associated with progressive hepatic fibrosis that is overly exuberant wound healing resulting in the overproduction and accumulation of extracellular matrix (ECM) proteins in the liver[7]. There are some evidences that myofibroblasts derived from hepatic stellate cells (HSCs) are the major sources of

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the abnormal ECM, including collagens type I and IV[8-10].

Interestingly, curcumin, the most active component of turmeric (*Curcuma longa* rhizomes), has potent antioxidant, anti-inflammatory, and wound healing properties[11-13]. Several studies have shown that curcumin can directly inhibit various inflammatory regulators. For instance, curcumin can reduce nuclear factor-kappa B (NF-kB) activation and decrease the production of cyclooxygenase (COX)-2 enzyme, which play an important role in the inflammatory cascade[2,13,14]. In addition, curcumin could attenuate the severity of hyperglycemia conditions, nephropathy, pancreatitis, and hepatic disease[15,16].

The purpose of the present study is to evaluate the potential effects of curcumin on the levels of inflammatory regulators; IL-13, TNF-alpha, and ED1 in the liver of type 1 diabetic rats. Moreover, this study also aims to investigate the effect of curcumin on inflammation-induced hepatic fibrosis by measuring the levels of ECM proteins, type I and type IV collagens.

Materials and Methods

Animals

Adult male Wistar rats (weight 200 to 250 g obtained from National Laboratory Animal Center of Mahidol University, Salaya, Thailand) were housed at least one week under a 12 h-light/dark cycle (25±2°C) with free access to standard pelleted chow and water. The study was performed in accordance with experimental protocols approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (under license No. 1/2557).

Induction of diabetes and animal groups

To construct the model of insulin-dependent experimental model, streptozotocin (STZ) was used as a diabetogenic agent owing to its ability to destroy pancreatic beta cells[17]. Diabetic rats were induced by a single intravenous injection of STZ (Sigma, St. Louis, MO, USA). Rats with blood sugar level >250 mg/dl were defined as diabetic animals. The animals were randomly divided into three groups:

Group I: Control group (C): the rats were injected with citrate buffer (0.1 M) alone.

Group II: Diabetic group (DM): the rats were injected with STZ (60 mg/kg BW). The STZ-induced animals were checked to have diabetic condition (DM). Then, DM group would be daily fed only with corn oil diet (3 ml/kg BW) by intragastric feeding.

Group III: Diabetic rats supplemented with curcumin (DMC): the diabetic rats were received daily fed with corn oil diet (3 ml/kg BW) containing curcumin (200 mg/kg BW, Sigma, St. Louis, MO, USA) by intragastric feeding.

Tissue preparation

All animals were observed and investigated up to 12 weeks. At the final point of 12 weeks, the rats from each groups were intensely anesthetized by isoflurane and quickly surgical terminated. The anteromedial lobe of liver was dissected, quickly frozen in dry ice, and stored at -80°C until use. For immunohistochemical staining, liver tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The liver tissues were sectioned (5 μm thick) and adhered onto positive charged coated slides.

Western blot analysis

The liver tissues were lysed in radio-immuno-precipitation assay buffer (RIPA buffer) (Santa Cruz Biotechnology, Inc) with a sonicator and then centrifuged at 12,000 g for 15 minutes at 4°C. The supernatants were collected for use in western blot analysis. The protein concentration was determined by the Bradford method. The bovine serum albumin (BSA) was used as a standard protein. The lysate proteins were separated by 10% (for type I and type IV collagen) or 15% (for IL-13 and TNF-alpha) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was incubated in blocking buffer (5% nonfat milk in PBST) for 1 h at room temperature. The membrane was then incubated with a mouse monoclonal antibody against actin (Millipore, MA, USA; 1: 1,000), mouse monoclonal antibody against IL-13, rabbit polyclonal antibody against TNF-alpha (Abcam, Cambridge, UK; 1: 1,000), goat polyclonal antibody against type I collagen, or type IV collagen (Santa Cruz, CA, USA; 1: 500) diluted in 3% BSA in PBST at 4°C for overnight. After washing with PBST, these membranes were incubated with 1: 10,000 horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, 1: 5,000 HRP-conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK), or 1: 7,500 HRP-conjugated donkey anti-goat IgG antibody (Santa Cruz, CA, USA) for 1.5 h at room temperature, respectively. Finally, protein bands were visualized by enhanced chemiluminescence using ECL Plus™ western blotting detection reagents and were exposed on x-ray film. The immunoblot bands were quantified by measuring the density of each band using densitometry with Scion image program (National Institutes of Health, Bethesda, MD). The optical density of the IL-13, TNF-alpha, type I and type IV collagen bands were normalized relative to the optical density of corresponding actin.

Immunohistochemistry for ED1

The immunohistochemical staining for ED1 was performed using mouse monoclonal antibody against of ED1 (Abcam, Cambridge, UK; 1: 50) and biotinylated goat anti-mouse IgG antibody (Santa Cruz, CA, USA; 1: 500) with avidin-biotin complex (ABC Elite, Vector). The protocol of this method has been described previously[18]. The immunoperoxidase activity was visualized by light microscopy (Olympus BH2, Tokyo, Japan). For semi-quantitative densitometric analyses of the immunoreactions, images were photographed with a digital camera (Olympus DP70, Tokyo, Japan; 8-bit color depth). Densitometric analysis of ED1-immunoreactivity was performed using cellSens Dimension Software (Olympus). The relative optical density to background staining was measured within selected areas.

Statistical analysis

All data were expressed as the mean ± SEM. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used in the present study to examine differences of the data. The significance levels were set at *p*-values less than 0.05.

Results

Effect of curcumin on diabetic-induced alteration of inflammatory regulators; IL-13 and TNF-alpha levels in the liver

To access the comparative IL-13 and TNF-alpha expressions among three groups of control (C), diabetes (DM) and diabetes supplemented with curcumin (DMC), the characteristic of both protein expressions in rat liver tissues was determined by western blot analysis. The results showed that the level of IL-13 in liver tissues of DM group increased significantly to 161.93±29.15% ($p<0.05$) of the control value, whereas the level in DMC group significantly decreased to 93.87±21.26% ($p<0.05$) compared with the level in the DM group (Figure 1).

The level of TNF-alpha in DM group significantly increased to 157.87±11.16% ($p<0.05$) of the control value. Treatment with curcumin significantly reduced the TNF-alpha level to 84.97±12.06% ($p<0.05$) compared with that in the DM group (Figure 2).

Effect of curcumin on diabetic-induced alteration of ED1-immunoreactivity in the liver

To determine the effect of curcumin on diabetic-induced inflammation in the liver, the marker of Kupffer cells, ED1, was examined by immunohistochemistry. ED1, a protein found in the granules of macrophages, appeared as a dot-like cytoplasmic or finely granular positivity by immunohistochemical methods. Localization and characterization of ED1 were compared among C, DM, and DMC groups.

Immunoexpression of ED1 in control group were rarely investigated along hepatic sinusoid (Figure 3A, B). In contrast, DM group showed the strong brown color intensity of ED1 appearance along hepatic sinusoid (Figure 3C, D). From semiquantitative analysis, the level of ED1-immunoreactivity in DM group significantly increased to 170.44±8.97% ($p<0.01$) compared with the level in the control group (Figure 4). In DMC group, ED1 was demonstrated as weak brown color intensity along hepatic sinusoid (Figure 3E, F) and ED1-immunoreactivity level

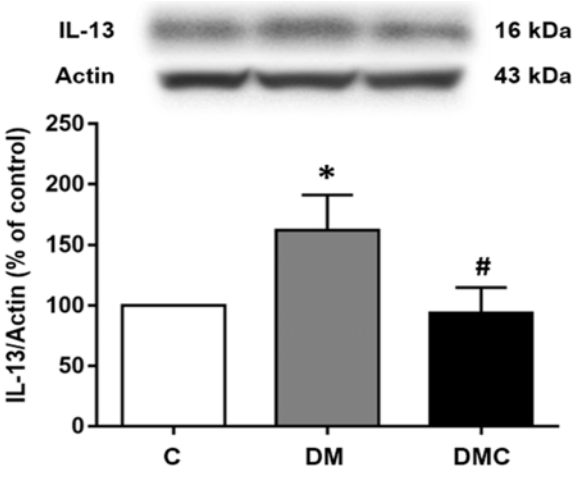

Figure 1. The effect of curcumin on diabetic-induced alteration in IL-13 proteins expression in the liver. The IL-13 protein levels of control group (C), diabetic group (DM) and diabetes supplemented with curcumin group (DMC) were measured by western blot analysis. Representative bands from different three groups were shown. The protein band was quantified via densitometry and normalized by actin. All western blot data were shown in the percentage of control (* *p*-value <0.05 compared with the control group; # *p*-value <0.05 compared with the DM group).

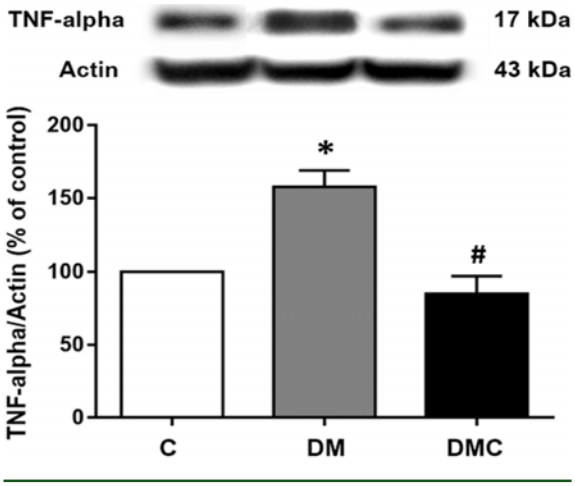

Figure 2. The effect of curcumin on diabetic-induced alteration in TNF-alpha proteins expression in the liver. The TNF-alpha protein levels of control group (C), diabetic group (DM) and diabetes supplemented with curcumin group (DMC) were measured by western blot analysis. Representative bands from different three groups were shown. The protein band was quantified via densitometry and normalized by actin. All western blot data were shown in the percentage of control (* *p*-value <0.05 compared with the control group; # *p*-value <0.05 compared with the DM group).

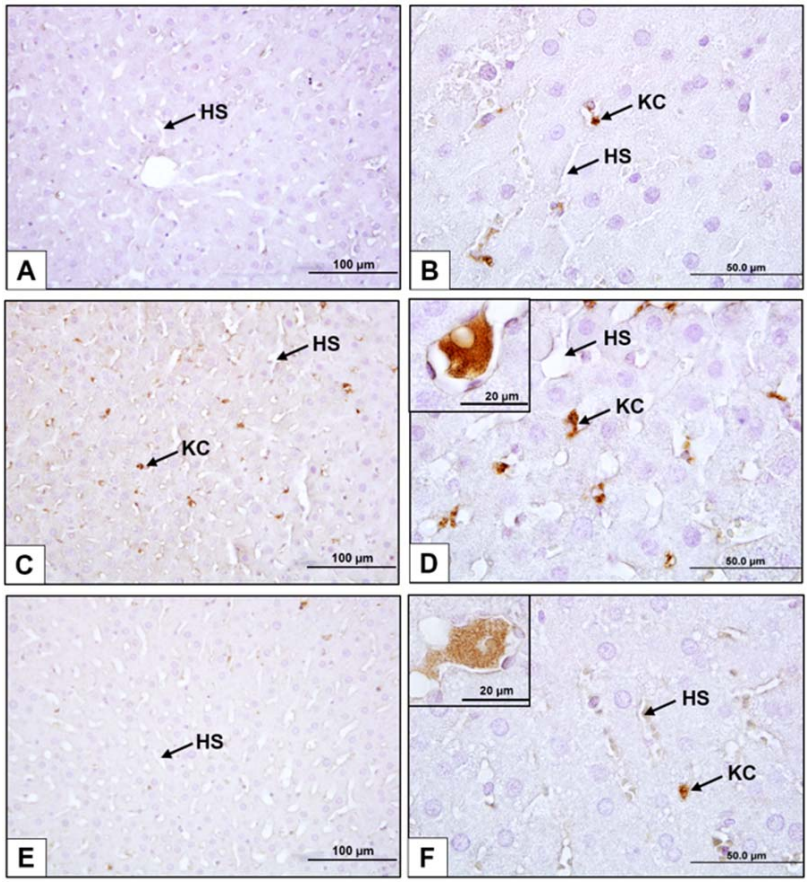

Figure 3. Micrographs of ED1 distribution in hepatic sinusoid of rat liver tissue, stained by immunohistochemical method. The immunoreactivity positive reaction was demonstrated among three groups of rats; control group (C): (3A, B), characterization of ED1 (arrow) was presented in the hepatic sinusoid with very weak brownish color. Diabetic group (DM): (3C, D), the intensity of brownish color of ED1 (arrow) was clearly increased in the hepatic sinusoid, compared with C group. Diabetes supplemented with curcumin group (DMC): (3E, F), the intensity of ED1 (arrow) was presented in the hepatic sinusoid with a little brownish color. A, C, E = 40x magnification, D, E, F = 100x magnification (insets show higher magnification of cytoplasmic immunoreactivity in macrophages; 150x), HS = hepatic sinusoid, KC = Kupffer cell.

significantly decreased toward to 111.52±7.05% (p<0.01) compared with that of DM group (Figure 4).

Effect of curcumin on diabetic-induced alteration of type I and type IV collagen levels in the liver

To determine the effect of curcumin on diabetic-induced hepatic fibrosis, the severity of hepatic fibrogenesis was estimated. Hence, the levels of ECM proteins, type I and type IV collagens in the liver can be used as the markers to assess hepatic fibrosis. Western blot analysis was used to identify specific collagen proteins from liver complex protein mixtures. The results showed that the level of type I collagen in DM group increased significantly to 156.60±33.99% (p<0.05) of the control value. However, the level of type I collagen in DMC group significantly decreased to 95.88±4.25% (p<0.05) compared with the level in the DM group (Figure 5). Consistent with type I collagen results, the level of type IV collagen in DM group increased significantly to 172.10±34.77% (p<0.05) of the control value, whereas the level in DMC group significantly reduced to 94.44±5.49% (p<0.05) compared with that in the DM group (Figure 6).

Discussion

In the present study, the major findings are described in the following criteria. Firstly, curcumin attenuated the liver inflammation through decreasing IL-13 and TNF-alpha. Secondly, curcumin administration inhibited the fibrogenesis, resulting in lower levels of type I and type IV collagen in the liver of diabetic rats. In these experiments, the levels of IL-13, TNF-alpha, type I collagen, and type IV collagen were markedly elevated in the DM group compared with that in the control group, but the levels of these proteins

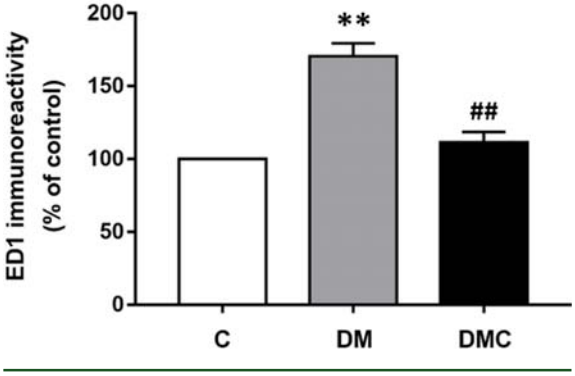

Figure 4. Semiquantitative densitometric analysis of ED1-immunoreactivity was performed in control group (C), diabetic group (DM) and diabetes supplemented with curcumin group (DMC). Data were shown in the percentage of control (** *p*-value <0.01 compared with the control group; ## *p*-value <0.01 compared with the DM group).

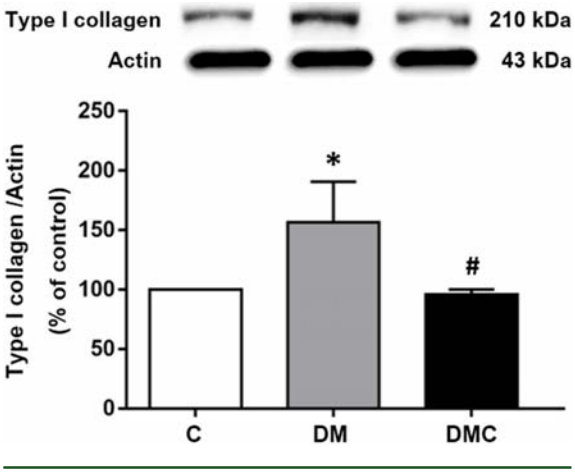

Figure 5. The effect of curcumin on diabetic-induced alteration in the level of type I collagen in the liver. The type I collagen protein levels of control group (C), diabetic group (DM) and diabetes supplemented with curcumin group (DMC) were measured by western blot analysis. Representative bands from different three groups were shown. The protein band was quantified via densitometry and normalized by actin. All western blot data were shown in the percentage of control (* *p*-value <0.05 compared with the control group; # *p*-value <0.05 compared with the DM group).

were significantly decreased in the DMC group compared with that in the DM group. The present results are consistent

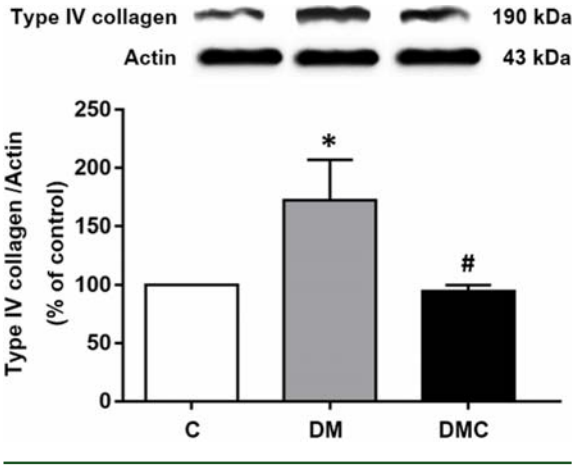

Figure 6. The effect of curcumin on diabetic-induced alteration in the level of type IV collagen in the liver. The type IV collagen protein levels of control group (C), diabetic group (DM) and diabetes supplemented with curcumin group (DMC) were measured by western blot analysis. Representative bands from different three groups were shown. The protein band was quantified via densitometry and normalized by actin. All western blot data were shown in the percentage of control (* *p*-value <0.05 compared with the control group; # *p*-value <0.05 compared with the DM group).

with our previous researches that examined the distribution of these proteins by immunohistochemical techniques[18,19]. Concerning this work, STZ was used to induce type 1 diabetes through its ability to selectively destroy pancreatic beta-cell[17]. It is well-known that type 1 diabetes is considered as an inflammatory process[20] that exhibits a significant increase of inflammatory cytokines such as IL-6, IL-18, IL-1[21] in the blood of patients. In the past decade, the liver injury has been recognized as a major complication of DM. The inflammatory response has been identified as one of the most important causes of hepatic injury in DM. The inflammatory process is associated with variations of plasma proteins and pro-inflammatory cytokines. Plasma concentrations of proteins are largely dependent on the hepatic biosynthesis, and changes in their production are influenced by pro-inflammatory cytokines such as IL-1, IL-6, and TNF-alpha[4]. The chronic hyperglycemia can accelerate the formation of AGEs and produce inflammatory cytokines in the liver. In the current study, the authors evaluate Kupffer cell, a hepatic macrophage, infiltration by ED1-immunohistochemical staining. As expected, the number of ED1-positive exudate macrophages showed a significant increase in diabetic rat compared to the control group. Additionally, it has been reported that Kupffer cells are the main source of TNF-alpha production and might act as an early trigger in the

genesis of hepatic fibrosis[22].

TNF-alpha is one of the major cytokines involved in alcoholic hepatitis, viral hepatitis, and ischemia/reperfusion in liver injury. Several studies have shown that TNF-alpha can either promote NF-kB activation or initiate the caspases activation pathway, which plays a major role in execution of apoptosis[8,20,21]. An early study has indicated that hyperglycemia-induced oxidative stress is associated with increased TNF-induced apoptosis in hepatocytes[14].

Besides TNF-alpha, IL-13 has been reported to play important roles in inflammatory and immune responses[23]. Previous studies demonstrated that IL-13 could alternatively activate macrophages, which have different roles in humoral immunity. Most recently, it was reported that IL-13 could control hepatic glucose production[5], which might cause the reactivity in up-regulation of IL-13 in the diabetic heart[24]. Moreover, IL-13 is also a key cytokine in the anti-inflammatory arm of the immune system. Nevertheless, the level of serum IL-13 is increased in subjects with metabolic syndrome[23], while could speculate that the anti-inflammatory cytokines, including IL-13, might have been activated to counteract excessive pro-inflammatory signaling. Additionally, the excess production of IL-13 can cause harm to the liver because IL-13 is well established as a critical molecule that directly activates HSCs, a primary cellular source of ECM components in hepatic fibrosis[8]. Moreover, HSCs are activated not only by IL-13 but also by other inflammatory cytokines, including TNF-alpha[6]. Subsequently, activated HSCs are changed and transdifferentiated to be myofibroblasts that produce a large amount of abnormal ECM, including collagens[8-10]. In vitro experiment reveals that IL-13 directly induces expression of collagens in HSCs[10]. Hepatic fibrosis is involved in major alteration in both quantity and composition of ECM, especially type I, III, and IV collagens.

The present results, therefore, revealed that both type I and type IV collagens are significantly elevated in diabetic-induced inflammatory livers. Type I collagen was proposed to associate with the feature of liver reorganization and was presented predominant deposition in the ECM in wounded liver[9]. There is some evidence that type I collagen fragment is elevated in hepatic fibrosis but not in patients with prostate, lung or breast cancer[25]. In this regard, type I collagen can be considered as a putative biomarker for hepatic fibrosis. Moreover, many studies have suggested that type IV collagen is the important surrogate maker of basement membrane formation and sinusoids capillarization, which are important pathological processes in hepatic fibrosis[9]. Therefore, the up-levels accumulation of both type I and type IV collagens in diabetic-induced liver might confirm the correlating with the feature of pathological hepatic fibrosis. Fascinatingly, curcumin administration successfully inhibited diabetic-induced inflammation by reducing the numbers of Kupffer cells, resulting in lower levels of IL-13 and TNF-alpha. In addition, the levels of type I and type IV collagens were obviously decreased in diabetic rats supplemented with curcumin. The low levels of collagens in DMC liver tissue could be implied that curcumin could inhibit diabetic-induced hepatic fibrosis. These beneficial effects of curcumin were hypothesized to be due to directly inhibition of various inflammatory regulator activity as well as indirectly influence inflammatory regulators through its ability to scavenge free radicals.

Conclusion

Many studies have emphasized the therapeutic activities of curcumin that they are associated with suppression of inflammation, angiogenesis, and diabetes. The present results support the hypothesis that curcumin can reduce hepatic inflammation and fibrosis in diabetic liver tissues. Therefore, dietary curcumin might have efficacy to ameliorate diabetic-induced hepatic injury in terms of anti-inflammatory and antifibrotic supplements. However, the exact mechanisms still require further investigation.

What is already known on this topic?

The characterization and localization of IL-13, TNF-alpha, type I and type IV collagens by immunohisto chemical techniques have been reported in the liver tissue of diabetic rat, but the detection method and duration of experiment are different from the present work.

What this study adds?

Although the antidiabetic properties of curcumin have been extensively studied, there are no quantitative data to establish the potential effects of curcumin on diabetic-induced hepatic fibrosis and subsequent recovery of diabetic liver tissue. The present study indicated the effects of curcumin on the levels of inflammatory regulators; IL-13, TNF-alpha, and Kupffer cells as well as the levels of type I and type IV collagens that could be used as the markers of hepatic fibrosis. The present results may serve as a tentative explanation for the effect of curcumin on the molecular pathway that links chronic liver inflammation with progressive hepatic fibrosis in the diabetic rat.

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Potential conflicts of interest

The authors declare no conflict of interest.

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