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The flavonoid quercetin inhibits dimethylnitrosamine-induced liver damage in rats

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Abstract

Quercetin, one of the most abundant flavonoids in human diet has been reported to exhibit a wide range of pharmacological properties. In this study, we investigated the protective effect of quercetin on hepatic injury induced by dimethylnitrosamine (DMN) in rats. Treatment with DMN caused a significant decrease in body and liver weight. Oral administration of quercetin (10 mg kg^{-1} daily for 4 weeks) remarkably prevented this DMN-induced loss in body and liver weight and inhibited the elevation of serum alanine transaminase, aspartate transaminase and bilirubin levels. Quercetin also increased serum albumin and hepatic glutathione levels and reduced the hepatic level of malondialdehyde. Furthermore, DMN-induced elevation of hydroxyproline content was reduced in the quercetin treated rats, the result of which was consistent with a reduction in type I collagen mRNA production and histological analysis of liver tissue stained with Sirius red. A reduction in hepatic stellate cell activation, as assessed by α-smooth muscle actin staining, was associated with quercetin treatment as well as a reduction in transforming growth factor-β1 expression. In conclusion, these results demonstrate that quercetin exhibited in-vivo hepatoprotective and anti-fibrogenic effects against DMN-induced liver injury and suggest that quercetin may be useful in the preventing the development of hepatic fibrosis.

Introduction

Hepatic fibrosis is a wound-healing response to chronic liver injury, which leads to cirrhosis and liver failure if repetitive liver damage occurs. Exciting progress has been made in the understanding of the mechanisms of hepatic fibrosis, which demonstrates that the central event of liver fibrosis is the activation of stellate cells involving the transformation of quiescent vitamin-A-rich cells into proliferative, fibrogenic and contractile myofibroblasts. Therefore, targeting the stellate cells and fibrogenic mediators could be one of the mainstays of anti-fibrotic therapy (Li & Friedman 1999). Quercetin is one of the most abundant dietary flavonoids and has been reported to exhibit a wide range of pharmacological properties, including hepatoprotective effects (Peres et al 2000; Knekt et al 2002). Recent studies have documented the inhibition of stellate cell activation and proliferation by quercetin, using rat hepatic stellate cells (HSCs) (Kawada et al 1998) and cell line HSC-T6 cells (Kang et al 2001). However, these effects of quercetin remain to be elucidated in-vivo. The purpose of our study was to determine whether treatment with quercetin exerts any beneficial effect in liver histopathology and liver function in-vivo. In this study, we evaluated the effects of quercetin on experimental hepatic fibrosis by dimethylnitrosamine (DMN) in rats.

Methods

Induction of liver fibrosis with DMN

Animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. Male Sprague-Dawley rats were obtained from Dae-Han Laboratory Animal Research Center Co., Ltd (Seoul, Korea). Animals were kept on standard rat chow

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with free access to tap water, in a temperature- and humidity-controlled animal house under 12-h light–dark cycles. Eighteen rats, 140–160 g, were divided into 3 groups of 6 each. The quercetin group was treated with intraperitoneal injections of DMN (diluted with saline) at a dose of 10 mg kg^{-1} per day for 3 consecutive days per week for 4 weeks (Jezequel et al 1989), and treated daily with quercetin (suspended in 0.5% carboxymethylcellulose sodium, CMC) at a dose of 10 mg kg^{-1} by oral gavage for the length of the study. The DMN group was treated with DMN as described above and equivalent volumes of 0.5% CMC solution. The control group was treated with the volumes of saline and 0.5% CMC solution equivalent to those of the quercetin group. At the end of the fourth week, all rats were sacrificed under ether anaesthesia and their livers were excised and weighed. Blood samples for biochemical analyses were obtained from the inferior vena cava. The liver specimens were either immediately frozen for hydroxyproline measurements or fixed in 10% neutral buffered formalin (NBF) for histochemical studies. The remaining liver tissue was homogenized using a glass Potter-Elvehjem homogenizer set. The homogenate was freed from the cellular debris and nuclei by centrifugation at 700 *g* at 4 °C for 10 min and was then centrifuged at 9000 *g* at 4 °C for 20 min and protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Biochemical analyses of serum

Serum aspartate transaminase (AST) and alanine transaminase (ALT) activity was estimated by colorimetric methods using commercial kits (Eiken, Tokyo, Japan) (Reitman & Frankel 1957). Serum albumin, total protein and bilirubin levels were also measured using commercial kits following the manufacturer's protocols.

Histology and immunohistochemistry

Five-micrometer liver sections were deparaffinized and processed routinely for haematoxylin-eosin (H&E) and Sirius red (SR) staining (Junqueira et al 1979), and were examined immunohistochemically for α-smooth muscle actin (α-SMA; Serotec, Oxford, UK) using routine indirect avidin-biotin immunolabelling procedures. Non-immune isotype-matched immunoglobulin was used in place of the primary antibody in the negative controls.

Hydroxyproline determination

Hydroxyproline levels in the liver were determined according to the Woessner method (Woessner 1961). Approximately 100 mg of liver tissue was hydrolysed in 6 M HCl in a sealed tube at 110 °C for 24 h. The hydrolysates were evaporated and the residues were dissolved in distilled water and filtered. The filtrate was then mixed with 1.4% chloramine-T solution and perchloric acid. Finally, 20% *p*-dimethylaminobenzaldehyde in methylcellosolve was added, and placed in a water bath at 60 °C for 20 min. After cooling, the absorbance was measured at 560 nm.

Determination of non-protein SH and malondialdehyde (MDA)

The Higashi method was used for the determination of non-protein SH (Higashi 1988). MDA levels were determined by the Buege & Aust (1978) method.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the liver using an RNA extraction kit (Intron, Seoul, Korea) following the manufacturer's protocol. Briefly, 50 mg of tissue sample was ground using a mortar and pestle at −70 °C and transferred to a tube containing 1 mL of the RNA extraction solution. The homogenate was then extracted with chloroform, precipitated with isopropanol, washed in ethanol and resuspended in 30 μl of autoclaved, double-distilled water. RNA concentration and purity were determined by absorbance at 260 and 280 nm. Samples exhibiting an absorbance ratio (260/280) greater than or equal to 1.7 and strong 28S and 18S ribosomal RNA bands on 1% (w/v) agarose gels were used for further analysis. RNA samples were stored at −80 °C until required.

First strand cDNA was obtained by reverse transcription (RT) using 5 μg of total rat liver RNA. The reaction was conducted in 20 μl of buffer containing 0.5 μg of Oligo $(dT)_{12\text{-}18}$ primer (Gibco-BRL, Grand Island, NY), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 40 mM DTT, 0.5 mM deoxynucleotide triphosphate (dNTP) mixture (Gibco-BRL), 10 U RNAse inhibitor (Gibco-BRL), and 200 U MMLV reverse transcriptase (Gibco-BRL). After incubation at 37 °C for 60 min, the reaction was stopped by heating at 70 °C for 15 min. To remove the remaining RNA, 1 μl of *Escherichia coli* RNase H (4 mg mL^{-1}) was added to the reaction mixture and incubated at 37 °C for 30 min. The cDNA was used as a template for PCR amplification using gene-specific primers for rat TGF-β1, Type 1 collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA primers (Bioneer, Cheongwon-Kun, Chungbuk, Korea) for each gene were designed, and consisted of forward primers (TGF-β1; 5′-GCC-CTG-GAT-ACC-AAC-TAC-TGC-T, Type I collagen; 5′-AAG-AAG-GCG-GCA-AAG-GTC-3′, GAPDH; 5′-AAC-TCC-CTC-AAG-ATT-GTC-AGC-3′), corresponding to nucleotides 1247–1266 (TGF-β1), 2827–2844 (Type I collagen) and 1266–1287 (GAPDH), and reverse primers (TGF-β1; 5′-AGG-CTC-CAA-ATG-TAG-GGG-CAG-G-3′, Type I collagen; 5′-GGA-CCT-TGT-TTG-CCA-GGT-3′, GAPDH; 5′-GGG-AGT-TGC-TGT-TGA-AGT-CAC-A-3′) complementary to nucleotides 1386–1407 (TGF-β1), 3062–3079 (Type I collagen) and 1693–1714 (GAPDH) in the rat TGF-β1, Type I collagen and GAPDH gene sequence (Genbank X52498, AF106860 and NM000088, respectively). These were used to amplify and to analyse the rat TGF-β1 (161 bp), Type I collagen (253 bp) and GAPDH (449 bp) transcripts. PCR amplification of the cDNA was performed in an automated thermal cycler (TECHNE, Teddington, UK) in a final volume of 25 μl containing 4 μl of cDNA solution, 20 mM Tris-HCl (pH 8.4),

50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTP mixture (Gibco-BRL), 0.4 pmol of each primer and 5 U of *Taq* DNA polymerase (Promega, Madison, WI). The amplification included an initial denaturation at 94 °C for 5 min and then 35 (TGF-β1 and Type I collagen) and 25 (GAPDH) cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min, ending with a 4 °C hold cycle. After the PCR, the amplified products were analysed by electrophoresis in 1.5% agarose gel (50% NuSieve, Tebu; 50% NA, Pharmacia Biotech, Uppsala, Sweden) and visualized by ethidium bromide staining under UV light illumination.

Statistical analyses

All values were expressed as the mean ± s.d. Significant differences between the control, DMN and DMN plus quercetin were statistically analysed using a one-way analysis of variance. Individual differences between the means of each treatment were compared using a Dunnett's test. A P value of ≤ 0.05 was considered statistically significant.

Results

Body and liver weight

The effects of quercetin on body weight and liver weight of rats are shown in Table 1. Treatment with DMN caused a significant decrease in both body weight and liver weight compared with the control group. Oral administration of quercetin tended to suppress the loss in body weight and significantly prevented the DMN-induced loss of liver weight (90% of control).

Serum parameters of liver function

The effects of quercetin on serum parameters in the liver fibrosis model are shown in Table 2. DMN-induced increases in serum AST, ALT and bilirubin were dramatically suppressed by quercetin treatment. In the case of chronic liver diseases, the serum albumin level is reduced due to protein synthesis disorder in the liver. Again, in the quercetin group, diminished serum albumin and total protein concentrations were restored to the control levels. These results indicate that quercetin protected the necrosis of hepatocytes due to DMN administration.

Histopathology and immunohistochemistry

The effects of quercetin on DMN-induced liver injury were evaluated by histopathologic examination of the liver sections by H&E staining. In contrast to the control group of rats (Figure 1A1), the dispensation of DMN for 4 weeks caused extensive haemorrhagic necrosis and disruption of tissue architecture (Figure 1A2). These alterations were remarkably reduced in the liver sections of quercetin-treated rats (Figure 1A3). Serial sections were stained with SR for collagen. In the liver sections taken from the control, almost no collagen fibres were observed except in the periportal area (Figure 1B1). The livers of

Table 1 Effect of quercetin on body and liver weights in dimethylnitrosamine-induced hepatic fibrosis in rats.

	Control	DMN	DMN + quercetin
Body weight (g)	282.0 ± 8.9	171.0 ± 9.7$^{###}$	226.5 ± 11.8***
Liver weight (g)	10.57 ± 0.53	5.62 ± 0.59$^{###}$	9.55 ± 1.23***

The values are expressed as means ± s.d. of six rats. DMN, 10 mg kg^{-1} DMN intraperitoneally alone; DMN + quercetin, 10 mg kg^{-1} DMN intraperitoneally with 10 mg kg^{-1} quercetin daily by oral gavage. $^{###}P < 0.001$ vs controls; ***$P < 0.001$ vs DMN group.

Table 2 Effects of quercetin on serum parameters of DMN-treated rats.

	Control	DMN	DMN + quercetin
AST (U L^{-1})	65.5 ± 6.5	641.1 ± 237.6$^{###}$	113.7 ± 14.3***
ALT (U L^{-1})	15.0 ± 2.7	235.1 ± 109.8$^{###}$	59.5 ± 14.1***
Albumin (g dL^{-1})	4.1 ± 0.1	2.1 ± 0.5$^{###}$	3.9 ± 0.1***
Total protein (g dL^{-1})	6.4 ± 0.3	4.4 ± 1.0$^{###}$	5.6 ± 0.3**
Bilirubin (mg dL^{-1})	0.17 ± 0.09	1.83 ± 0.67$^{###}$	0.57 ± 0.32***

AST, aspartate transaminase; ALT, alanine transaminase. Data represent the means ± s.d. of six rats. $^{###}P < 0.001$ vs control; **$P < 0.01$ and ***$P < 0.001$, vs the DMN group.

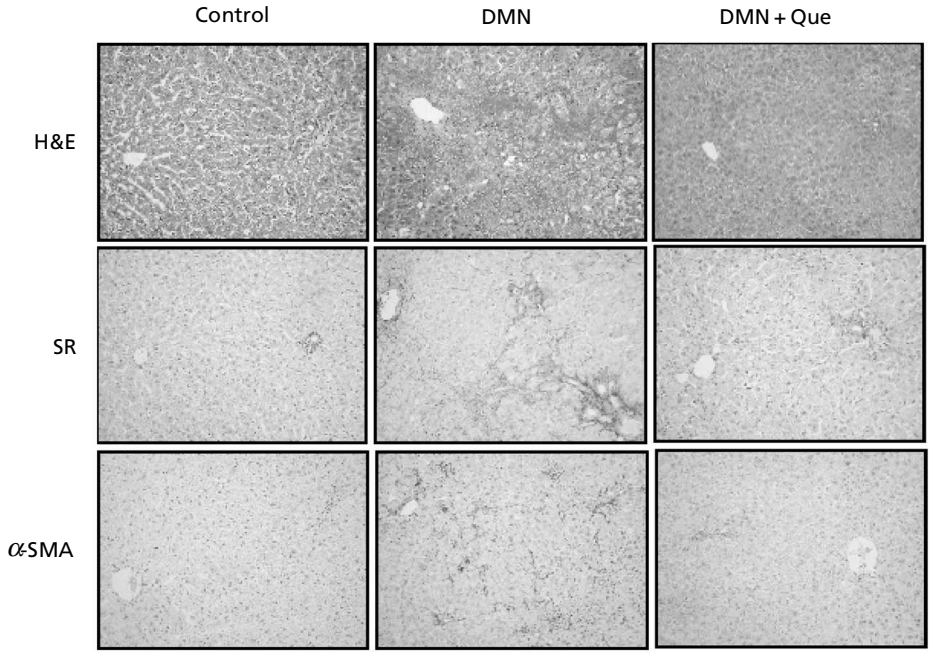

Figure 1 Histological analysis of rat liver sections. Liver samples were taken from the non-treated control rats (control), the rats treated with DMN (10 mg kg^{-1} per day for 3 consecutive days of each week for 4 weeks; DMN) and rats treated with DMN and quercetin (10 mg kg^{-1}, p.o. daily; DMN + Que). The sections were stained with haematoxylin–eosin (H&E) and with Sirius red (SR). A1. Control. A2. Haemorrhagic necrosis and distorted tissue architecture are seen. A3. Haemorrhagic necrosis is rarely observed and tissue architecture appears to be similar to that of control rats. B1. Control. B2. Thick fibrotic septa are seen. B3. Only incomplete fibrotic septa are observed. Activated HSCs were detected by immunohistochemistry with α-SMA antibody (α-SMA). C1. Control. C2. Remarkably increased α-SMA positive cells in number are seen throughout the parenchyma. C3. The numbers of α-SMA positive cells are markedly reduced.

DMN-treated rats exhibited an increase in collagen content, and displayed bundles of collagen fibres surrounding the lobules, forming large fibrous septa (Figure 1B2). The thickening of these collagen fibre bundles (stained in red) was markedly reduced in the quercetin group (Figure 1B3). The expression of α-SMA, an indicator of activated HSCs (Gressner 1996; Friedman 2000), was detected by the immunohistochemistry method. In contrast to the control group (Figure 1C1), many α-SMA-positive cells were detected around the periportal fibrotic band areas and were scattered in the regions of connective tissue septa in the DMN-treated rats (Figure 1C2). However, quercetin prevented the activation of most HSCs and, therefore, only traces of α-SMA positive cells were detected. The level of α-SMA expression was almost the same as in the liver of the control (Figure 1C3).

Hepatic hydroxyproline, non-protein SH content and lipid peroxidation

Liver fibrosis was also quantified by the measurement of hepatic hydroxyproline. DMN caused a 2.8-fold increase in hepatic hydroxyproline content, compared with the control group. However, quercetin treatment attenuated the DMN-induced increase in hepatic hydroxyproline content by 142% of the control (Table 3). This suggests that quercetin treatment suppressed the collagen deposition in liver fibrosis. The lipid peroxidation level in the liver was measured as MDA

Table 3 Effects of quercetin on the hydroxyproline, non-protein SH and malondialdehyde (MDA) contents in the liver of rats treated with DMN.

	Control	DMN	DMN + quercetin
Hydroxyproline (μg/0.1 g liver)	30.8 ± 7.0	85.1 ± 18.0$^{\#\#\#}$	43.7 ± 5.4***
MDA (nmol (mg protein)$^{-1}$)	0.51 ± 0.04	0.75 ± 0.14$^{\#\#\#}$	0.49 ± 0.02**
Non-protein SH (nmol (mg protein)$^{-1}$)	49.1 ± 0.9	36.0 ± 7.0$^{\#\#}$	45.2 ± 4.6*

Data represent the means ± s.d. of six rats. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, vs control; $^{*}P < 0.05$ and $^{**}P < 0.01$, vs the DMN group.

determination in homogenate. As shown in Table 3, the elevated MDA content in DMN-treated rats was remarkably reduced with quercetin treatment. Furthermore, the reduced non-protein SH content in DMN-treated rats was reversed with quercetin administration.

Production of TGF-β1 mRNA and type I collagen mRNA

TGF-β1 is one of the key mediators for fibrogenesis. DMN treatment increased the production of TGF-β1 mRNA, but quercetin reduced the production of TGF-β1 mRNA in the DMN-treated rat liver (Figure 2). Data indicated quercetin reduced TGF-β1 mRNA expression in the DMN-treated rat liver, which agrees with the fibrosis reduction. Next, we examined the effect of quercetin on type I collagen mRNA expression in the DMN-treated rat liver. Furthermore, DMN-induced elevation of type I collagen mRNA production was reduced in the quercetin-treated rats, the result of which was consistent with a reduction in hydroxyproline content and histological analysis of liver tissue stained with SR.

Discussion

Hepatic fibrosis represents a common response to chronic liver injury of various origins (e.g. viral, metabolic and toxic). Regardless of the type of insult, liver fibrosis is characterized by the increased production of extracellular matrix (ECM) proteins. In this study, quercetin significantly suppressed the increase in tissue hydroxyproline content in the DMN-induced hepatic fibrosis model in rats. The HSCs are activated in hepatic fibrosis. Activated HSCs have been identified as the primary source of excess ECM accumulation in liver fibrosis. Our present data show that DMN increased the number of α-SMA-positive cells in the liver and these proliferations are suppressed by quercetin ingestion. Taken together, these findings suggest the antifibrotic effect of quercetin may be due, at least in part, to the suppression of HSC activation, which is consistent with the suggestions in recent research, in which rat cultured HSCs (Kawada et al 1998) and cell line HSC-T6 cells (Kang et al 2001) have been studied.

Hepatic fibrogenesis is accompanied by hepatocellular necrosis and inflammation. HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver (George et al 1999; Li & Friedman 1999). Hepatocytes and Kupffer cells are potent sources of reactive oxygen intermediates and these compounds exert paracrine stimulation of stellate cells (Maher 1999). In this study, quercetin protects the hepatocytes from injury and improves the liver function of the DMN-treated rats. The hepatoprotective effects of quercetin may decrease paracrine stimuli, which lead to hepatic fibrosis via activated HSCs. TGF-β1 is a well-characterized cytokine that appears to play a major role in directing the cellular response to injury, driving fibrogenesis and, thus, potentially underlying the progression of chronic injury to fibrosis (George et al 1999). Up-regulation of TGF-β1 is well documented in experimental and human hepatic fibrosis (Castilla et al 1991; Lee et al 1995). This study shows that TGF-β1 mRNA expression in the liver of DMN-treated rats is significantly inhibited by quercetin. This indicates that the antifibrotic effect of quercetin may be associated, at least in part, with the inhibition of TGF-β1 production.

Oxidative stress plays an important role in many types of acute liver injury (Kadiiska et al 2000; Zhu & Fung 2000). Many experimental and clinical data indicate that a common link between chronic liver damage and hepatic fibrosis may be represented by oxidative stress (Peres et al 2000; Vendemiale et al 2001), which recently has been reported to be associated with the HSC activation (Lee et al 1995; Tsukamoto et al 1995). There is sufficient evidence suggesting that lipid peroxidation can occur in both

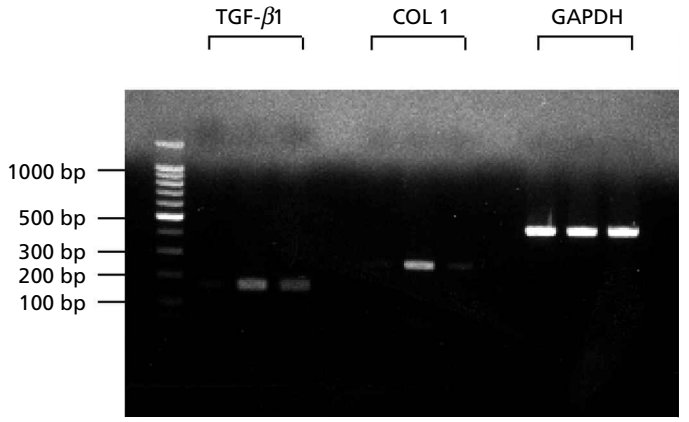

Figure 2 RT-PCR analysis of TGF-β1 and Type I collagen (COL 1) mRNA in homogenates of liver from untreated control rats (control) or rats treated with either dimethylnitrosamine alone (DMN) or DMN plus quercetin (DMN + quercetin). Ethidium bromide-stained agarose gel electrophoresis showing a representative RT-PCR result of co-amplification of TGF-β1 (161 bp), Type I collagen (253 bp) and GAPDH (449 bp). M, DNA size marker; 1, control; 2, DMN; 3, DMN + quercetin.

acute and chronic liver injury. Several studies have shown that lipid peroxidation stimulates collagen production in fibroblasts and HSCs and it plays an important role in the development of liver fibrosis (Parola et al 1993). GSH offers one of the mechanisms for the scavenging of toxic free radicals and GSH precursors have been shown to exert protective effects against HSC activation (Gasso et al 1996). In this study, DMN-treated rats exhibited an impaired oxidative balance (increased level of liver MDA and decreased level of liver GSH). However, quercetin restored this impairment. These outcomes suggest that the mechanism for the hepatoprotective effects of quercetin in the development of liver fibrosis may be related to the reduction of oxidative stress.

In summary, this study demonstrated that quercetin exhibited in-vivo hepatoprotective and antifibrotic effects against liver injury induced by DMN. The mechanism appeared mostly to be mediated by inactivation of HSCs but could also involve inhibition of TGF-β1 production. In addition, quercetin might also possess a beneficial effect on the restoration of impaired oxidative balance as an efficient antioxidant, which exerts a protective effect against HSC activation. Targeting the stellate cells and fibrogenic mediators could be one of the mainstays of anti-fibrotic therapy. Our data suggests that quercetin, one of the most abundant flavonoids in the human diet, may be potentially useful in the prevention of the development of hepatic fibrosis.

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