Administration of silymarin could promote the expression of proliferating cell nuclear antigen during liver regeneration induced by partial hepatectomy in rats

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ABSTRACT

Silymarin, a mixture of flavonolignans isolated from medicinal plant Silybum marianum, exhibits anti-carcinogenic, anti-inflammatory, and cytoprotective effect. Several reports have demonstrated the hepatocyte protective activity of silymarin. However, effect of silymarin on the expression of proliferating cell nuclear antigen (PCNA), an indicator of cell proliferation, is not fully determined. In this study, the effect of silymarin on the expression of PCNA in regenerating rat liver after 70% partial hepatectomy (PH) was investigated. In silymarin feeding (25 mg/kg) group before and after PH, the process of liver regeneration including collapse and rearrangement of hepatic plates and remodeling of sinusoids was more or less facilitated. In immuno-histochemical analysis, the expression of PCNA was much higher in the early stage of the experimental group. Western blotting analysis revealed that PCNA protein was more expressed in the early stage of the experimental group. RT-PCR results showed that the expression of PCNA-mRNA in liver tissue was also increased in the early stage of the experimental group. PCNA proteins labeled by gold particles were mostly located at the nucleolus and heterochromatin of nucleus in hepatocytes. These results suggest that silymarin could stimulate PCNA expression, probably promote DNA synthesis in the nucleus and accelerate the hepatocyte proliferation in regenerating rat liver induced by PH.

Key words: Silymarin, proliferating cell nuclear antigen, liver regeneration, partial hepatectomy.

INTRODUCTION

Liver is an important organ. It is involved in detoxification, carbohydrate metabolism, protein synthesis, blood clotting, and biochemical protection. It also plays an essential role in maintaining metabolic homeostasis and balance. The main cell type of the liver that carries out most of these functions is the parenchymal cell or hepatocyte that makes up about 80% of hepatic cells. Other cell types of the liver include non-parenchymal cells such as endothelial cells, Kupffer cells, lymphocytes, and stellate cells. All these cell types are activated by hepatic injury. Stellate cells particularly produce most factors that lead to hepatic fibrosis.

Liver is an excellent organ for growth regulation when it is damaged by physical stimulus such as partial hepatectomy (PH). Hepatocytes can regenerate through a process of compensatory growth to return to their original volume and quiescent state. Higgins and Anderson (1931) described a method to perform 2/3 partial hepatectomy in rats. Since then, liver regeneration by PH has been widely used to investigate liver response after injury and the regulatory mechanisms.

Liver regeneration is a successive response accompanied by sequential changes in gene expressions, cytokine productions, and morphological structures. After liver is damaged by external stimulus, hepatic plates consisting of
hepatocytes collapse and most mature hepatocytes begin to proliferate. It has been reported that within 24 h after regeneration, hepatocytes activate TGF-α, FGF, and VEGF, hepatic stellate cells do TGF-β1 and HGF while Kupffer cells do TNF and IL-6. After 24 h, DNA synthesis reaches its peak, and parenchymal cells proliferate followed by non-parenchymal cells re-establishing hepatic plates. Growth factors, insulin, cytokines, hormones, and proteases are involved in this process to facilitate liver regeneration (Fausto et al., 2012).

Proliferating cell nuclear antigen (PCNA) was originally identified as a nuclear protein whose appearance was correlated with the proliferative state of cell through immuno-fluorescence. PCNA, a cofactor of DNA polymerase δ, is highly preserved. Evolution from prokaryotes to eukaryotes involves changed function of PCNA from an executive molecule that controls critical cellular division pathways. PCNA is known to be a 36 kD non-histone nucleoprotein called cyclin found in the last 5% of G1 phase and the first 35% of S-phase of the cell cycle. It is crucial for DNA synthesis. PCNA contributes to the DNA repair process when DNA is impaired. PCNA protein has been extensively used as an indicator of proliferation because it has distinct increase in proliferating cells (Tanno and Taguchi, 1999; González et al., 2000).

Silymarin, the key chemical of this study, is a polyphenolic flavonoid antioxidant derived from Compositae (milk thistle, Silybum marianus). It is mainly composed of silibinin (approximately, 80%, w/w; also called silybin, silibin, or sibilinin) with smaller amounts of other stereoisomers such as isosilybin, silydianin, and silychristin (Salmi and Sarna, 1982; Valenzuela et al., 1986).

Silymarin is known to have strong antioxidative properties because it can scavenge both free radicals and reactive oxygen species (Kropáčová et al., 1998; Farghali et al., 2000; Kohno et al., 2002). It can inhibit the activation of Kupffer cells and hepatic fibrosis. Toxic substances on the membrane of hepatocytes can bind to silymarin receptor and stabilize cell membrane, and so hepatocytes can be protected. The main therapeutic effects of silymarin include accelerating the regeneration of hepatocytes, antioxidative activity, detoxifying activity, and suppressing hepatic fibrosis and inflammations (Parés et al., 1998; Clichici et al., 2015).

In recent years, silymarin has also been used in Asia as a therapeutic agent for liver diseases. Silymarin is well tolerated without much adverse effect. In Europe, silymarin has been clinically used as an anti-hepatotoxic for over 20 years to treat alcoholic-liver disease (Fehér and Lengyel, 2008).

There is a remarkable similarity in the process of hepatic regeneration between laboratory animals and humans. Thus, it is reasonable to assume that mechanisms regulating regeneration might be fairly similar among species. Knowledge gained from studies of liver regeneration in rats and mice is applicable to human liver. For several years, techniques and treatments for liver transplantation, cancer, and cirrhosis have been studied.

In the process of regeneration after hepatic injuries, humans are prone to complications such as hepatic failure and sepsisemia as well as death. Up to now, many studies have tried to find ways to facilitate liver regeneration and prevent complications following hepatic injuries (Yoon et al., 2015; Clichici et al., 2015). However, whether silymarin could promote the expression of proliferating cell nuclear antigen (PCNA) in regenerating rat liver induced by partial hepatectomy (PH) has not been reported. Therefore, the objective of this study was to determine the effect of silymarin on the expression of PCNA in rats during liver regeneration induced by PH.

MATERIALS AND METHODS

Animals and treatment

Male Sprague-Dawley rats weighting 210 to 230 g (7 weeks old) were housed in a 12 h light/dark cycle with free access to standard food and water. Procedures involving all animals and their care were conducted in conformity with the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Committee of Soochunhyang University. To establish partial hepatectomized model, 70% partial hepatectomy (PH) involving the median and left lateral lobes accounting for 40 and 30% of liver volume was performed according to the procedure of Higgins and Anderson (1931) under ethyl ether anesthesia (Madrahamov et al., 2006).

Hepatectomized rats were randomly divided into two groups: control group (24 rats) with oral administration of saline, and experimental group (24 rats) with silymarin (25 mg/kg). Silymarin or saline were orally administered for 7 days before and after 3 days of PH. Body weights were recorded daily. Animals were sacrificed at 0.5, 1, 2, 3, 5, and 7 days after PH. After the weights of body and liver were recorded, remnant liver tissues of rats were collected for light microscopy, immuno-histochemistry, western blotting, PCR, and immuno-cytochemical analysis of proliferating cell nuclear antigen (PCNA) (Figure 1).

Light microscopy and the immuno-histochemical analysis of PCNA

For light microscopy, liver tissues extracted from rats were fixed in 10% neutral buffered formalin for two days and dehydrated with graded alcohol series. Following xylen treatment, specimens were embedded in paraffin blocks, cut into 4 µm-thick sections using microtome (Leica Co. Germany), and placed onto slide glass. The sections were stained with hematoxylin and eosin (H-E) and mounted with Canada balsam. Histological observation was
Figure 1. Light micrographs of liver tissue at various days after 70% partial hepatectomy in rats of the control (saline treated) group (A, C, E, G, I, K) and the experimental (silymarin treated) group (B, D, F, H, J, L). CV (central vein). H-E stain. Scale bar indicates 400 µm.
performed using a microscope (IX51, Olympus Co. Japan). For immuno-histochemistry to localize and PCNA determined between control and experimental groups using LSAB+System-HRP Kit (Dako Co. USA), paraffin embedded sections were hydrated and washed with phosphate buffered saline (PBS). Sections were incubated with 3% hydrogen peroxide (diluted in PBS) for 5 min to block endogenous peroxidase activity. Non-specific binding sites for antibodies were blocked by incubating the sections for 15 min with 3% bovine serum albumin. Sections were incubated with primary antibody (1:200, PCNA mouse monoclonal antibody, Millipore Co. USA) at 4°C overnight and washed thrice with PBS. Washed samples were treated with biotinylated secondary antibody, incubated with streptavidin-HRP for 15 min, and washed in PBS. Sections were then stained with DAB (3, 3’-diaminobenzidine) for 3 min. All steps were carried out at room temperature in a humidified chamber to prevent specimens from drying. To quantify the PCNA expression among the groups, a double blind test was conducted to count and compare the PCNA positive cells (Figure 2).

**Western blot analysis for PCNA**

Rat liver tissues were homogenized and lysed with protein extraction solution (PRO-PREP™, Intron Co. Korea). Proteins were then measured using a Nanodrop-100 spectrophotometer (Thermo Co. USA). A total of 30 µg proteins per lane were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 4°C and 100 V for 150 min (Figure 3). Proteins were transferred onto PVDF membrane (hydrophobic polyvinylidene fluoride, GE Healthcare Co. UK) at 4°C and 250 mA for 50 min. Membranes were blocked with 10% skim milk for 1 h and incubated with primary PCNA antibodies (1:2,000, PCNA mouse monoclonal antibody, Millipore Co. Germany) and beta-actin (1:6,000, beta-actin mouse monoclonal antibody, Sigma Co. USA) diluted in 1% skim milk in PBS at 4°C overnight. After washing thrice in PBS, membranes were incubated with secondary antibody (1:3,000, goat anti-mouse IgG (H+L) HRP conjugate, Thermo Co. USA). Protein bands were then enhanced with chemiluminescence (WEST-ZOL, Western Blot Detection System, Intron Co. Korea). PCNA protein signals were recorded using Chemi-doc (Sygen Co. UK).

**Reverse transcription-PCR for PCNA-mRNA**

Freeze-dried liver tissues were lysed with lysis buffer. Total RNA was extracted using RNA extraction kit (Ribospin™, GeneAll Co. Korea). RNA was quantified on a Nanodrop-100 spectrophotometer. cDNA synthesis kit (iScript™ Select cDNA Synthesis Kit, Bio-rad Co. USA) and CFX96™ (CFX96™ RealTime System, Bio-Rad, USA) were used for cDNA synthesis. cDNA was quantified. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using CFX96™. RT-PCR products were subjected to 1% agarose (Sigma Co. USA) gel electrophoresis at 100 V for 30 min. PCNA and GAPDH band signals were then detected using Chemi-doc.

**Electron microscopic immunogold labelling for PCNA**

Liver tissues were cut into pieces (1 mm³) and fixed in 4% glutaraldehyde in PBS (0.1 M, pH 7.4) for 4 h. After rinsing in PBS, samples were dehydrated in graded alcohol series, substituted with propylene oxide (Acros Co. USA), embedded in araldite resin, cut into 80 nm in thickness with ultramicrotome (Reichert Supernova Ultramicrotome, Leica Co. Germany), and collected using 150 mesh-nickel grids. For antigen retrieval, samples were incubated in 0.01 M citrate buffer (pH 6.0) at 95°C for 15 min, treated with 1% BSA in EM-immunogold (EMG) buffer (0.05% Tween 20, 0.5 M NaCl, 0.01 M PB, pH 7.4) for 1 h to block non-specific reactions, and incubated with primary antibody to PCNA (1:50, PCNA mouse monoclonal antibody, Millipore Co. Germany) diluted in 0.1% BSA in EMG buffer at 37°C for 3 h. After rinsing twice in EMG buffer for 20 min, samples were incubated with 15 nm gold particle-conjugated secondary antibody (1:100, goat anti-mouse IgG) diluted in EMG buffer for 2 h, rinsed in 0.1% BSA in EMG buffer twice, and dried. Dried samples were oxidized in 0.1% OsO₄ in PBS for 1 min, stained with uranyl acetate for 3 min, and observed under transmission electron microscope (JEM-1010, JEOL Co. Japan) at 80 kV (Yoon et al., 2015). Labeled gold particles were counted under the microscope to compare their densities.

**RESULTS**

**Light microscopy and the immuno-histochemical observation of PCNA**

To investigate the effect of silymarin on histological changes of regenerating rat liver induced by partial hepatectomy (PH), double staining of liver sections with hematoxylin-eosin was performed. On light microscopy, the liver tissues of normal rat were typically composed of hepatic lobules, the structural units of liver. In the middle of each lobule, there was a central vein. At the periphery, there were branches of hepatic portal vein and hepatic artery, both of which were drained into the sinusoids between hepatic plates composed of hepatocytes. At 1 day after PH, hepatic plates began to collapse in both the control (saline treated) group and the experimental (silymarin treated) group. However, the control group seemed to progress more rapidly. At two days after PH, hepatocytes seemed to separate from each other and start to proliferate. At three
Figure 2. Immuno-histochemistry of PCNA in liver tissues of rats at various days after 70% partial hepatectomy in the control (saline treated, A-C-E-G-I-K) group and the experimental (silymarin treated, B-D-F-H-J-L) group. Scale bar indicates 400 µm.

days after PH, the dividing features of hepatocytes were markedly observed and the rearrangement of hepatic plates began to appear. At five days after PH, hepatic plates started to re-establish in both groups. In a later stage, most hepatic plates and sinusoids were reconstructed in the experimental group. However, they only made partial and slow progress in the control group. These results showed that silymarin treatment accelerated sequential changes
Figure 3. Western blot analysis of beta-actin and PCNA of liver homogenate. Liver protein (30 µg) was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 4. Proliferating cell nuclear antigen (PCNA) labelling index. The index was determined as the number of PCNA-positive cells per X400 microscope field. Each value and vertical bar represents mean ± SD.

(collapse of hepatic plates, cell proliferation, and reconstruction of hepatic plates and sinusoids) of liver regeneration induced by PH. Therefore, silymarin seemed to be able to accelerate the process of histological reconstruction in liver regeneration.

To determine whether silymarin treatment could affect the proliferation of hepatic cells in regenerating liver after PH, immuno-histochemistry using anti-PCNA was carried out. There were many PCNA staining-positive cells in regenerating rat liver after PH with strong staining intensities in the nuclei of hepatocytes (Figure 4). However, there were only weakly stained cells in the liver of normal rats. In the experimental group, positive reactions for PCNA were observed strongly in the nuclei and moderately strong
Figure 5. Relative densities of PCNA expression in the control group and the experimental group at various days after partial hepatectomy based on western blot analysis.

in the cytoplasm at 12 h and a day after PH. This staining pattern was less prominent in the control group and later stages of regeneration after PH. From day 5 after PH, PCNA expression gradually decreased. At day 7 after PH, the intensities of PCNA reactions were similar between the control group and the experimental group. The numbers of PCNA positive hepatocytes in silymarin administered rats were significantly more at 12 h and day 1 after PH than in the control group.

Western blot analysis for PCNA protein

Liver tissues extracted at each time point were subjected to western blot analysis to detect PCNA protein. House-keeping protein β-actin was used as a loading control. In the control (saline-treated) group, PCNA was increased gradually at 1 day after PH with peaked expression at 3 days after PH (Figure 5). Its amount was decreased thereafter. However, in the experimental (silymarin-treated) group, PCNA showed the highest amount at day 1 and 2 after PH followed by a sharp decrease at the 3rd and 5th day after PH. Quantitatively, the protein level of 1 day after PH in the experimental group was approximately 3.7 fold higher than that of the control group (relative density at 64.4 and 17.4 in the experimental group and the control group, respectively). This result suggested that silymarin promoted the expression of PCNA at the early stage and boosted the proliferation of hepatocytes and liver regeneration after PH.

Reverse transcription-PCR for PCNA-mRNA

Liver tissues from the control group and the experimental group were subjected to mRNA extraction and RT-PCR to determine the expression of PCNA-mRNA. In the control (saline-treated) group, the amount of PCNA-mRNA began to increase at 12 h, peaked at 1 day but decreased at 7 days after PH (Figure 6). In the experimental (silymarin-treated) group, the amount of PCNA-mRNA already increased at 12 h and 1 day after PH. This quantity was maintained at high level at 3 days but decreased significantly at 5 and 7 days after PH. This result demonstrated that silymarin stimulated the regeneration process by promoting the expression of PCNA-mRNA at the early stage after PH.

Electron microscopic immunogold labelling for PCNA

To determine the localization of PCNA in hepatocytes of regenerating rat liver, PCNA protein was ultra-microscopically investigated using the immunogold labelling method. Under transmission electron microscopy,
Figure 6. Reverse transcription-PCR of GAPDH-mRNA and PCNA-mRNA expression of liver homogenate supernatants in the control group and the experimental group at various days after partial hepatectomy.

gold particles indicating PCNA positive reactions were mostly localized in the heterochromatin and the nucleus of proliferating hepatocytes (Figure 7). More gold particles were observed in the experimental group as compared to the control group, especially at 1 day after PH (Figure 8). A few gold particles were in the cytoplasm of cells at the early stage of the experimental group. Such gold labelling pattern in the control and experimental groups was similar to results of immuno-histochemical observation.

DISCUSSION

Liver regeneration induced by partial hepatectomy (PH) is a process of compensatory growth. It starts active cellular division 24 h after surgery and stops when the original mass is restored. Silymarin is a flavonolignan compound derived from Compositae. It can protect hepatocytes by activating glutathione system, promotes protein synthesis and regeneration of damaged hepatocytes, and prevent hepatic fibrosis by suppressing activation of hepatic stellate cell and expression of cytokines (Mayer et al., 2005). Since proliferating cell nuclear antigen (PCNA) has been known as an indicator of cell proliferation, we focused on the effect of silymarin on the expression of PCNA protein in terms of liver regeneration induced by 70% PH in rat model. Animal groups were randomly divided into control (saline) group or experimental (silymarin, 25 mg/kg) group. Through a series of experiments, it was discovered that the rate of liver regeneration seemed to be faster in the early stage of the experimental group compared to the control group. However, the morphology of liver lobules were similar between the two groups. Since there was no difference in the activities of serum enzymes (ALP, AST, and ALT) related to liver function between the two groups (data not shown), silymarin might have few adverse effects on the liver.

In histological observation, the process of liver regeneration including collapse, proliferation and rearrangement of hepatic plates, and remodeling of sinusoids was facilitated in the experimental group. Immuno-histochemical, immuno-cytochemical, western blotting, and RT-PCR analyses revealed that the expression of PCNA was much higher in the early stage of liver regeneration of the experimental group (Figure 9). Taken together, these results suggest that silymarin could affect the expression of PCNA and histological re-establishment of liver during liver regeneration through regulation of different signal interactions between non-parenchymal cells and hepatocytes to accelerate liver regeneration (Paunesku et al., 2001). Silymarin can activate hepatic stellate cell, promote the expression of HGF, increase HGF, stimulate c-met receptor, and induce the growth and proliferation of hepatocytes through various signal pathways (Sozmen et al., 2014).

As previously mentioned, silymarin is one well-known plant extract for oral treatment of toxic liver damage. Silymarin can support liver cells through multifactorial actions, including binding to cell membranes to inhibit toxin penetration into hepatic cells, increasing SOD activity, and enhancing hepatocyte protein synthesis (Müzes et al., 1991). Recently, silymarin was found to possess antioxidant properties due to the phenolic nature of flavonolignans. Silymarin can act as a stimulant for liver cell proliferation and cell membrane stabilization (Sozmen et al., 2014) and accelerate the cell cycle (Wu et al., 2015). It was reported that silymarin-mediated cyclin D1 down-regulation might have resulted from proteosomal degradation through its threonin-286 phosphorylation through NF-kB activation (Eo et al., 2015), and that silymarin showed anti-proliferative, anti-inflammatory and energy antioxidant effects during hepatic regeneration in Wistar-Albino rats (Cetinkunar et al., 2015). However, the exact mechanism by
Figure 7. Immuno-cytochemical electron micrographs of PCNA in hepatocytes of the control group and the experimental group at various days after partial hepatectomy. Arrows: PCNA-gold particles. Scale bar indicates 500 nm.
Figure 8. PCNA-gold particles detected in the nuclei of hepatocytes per X20,000 viewing field in the control group (A, C, E, G) and the experimental group (B, D, F, H) at various days after partial hepatectomy. Each value represents mean ± SD (P>0.05).

Figure 9. Relative densities of PCNA-mRNA expression in the control group and the experimental group at various days after partial hepatectomy based on reverse transcription-PCR.
which silymarin administration promotes the PCNA expression in regenerating liver after PH remains to be uncovered.

CONCLUSION

Silymarin has been investigated for use as an antioxidant and anticarcinogen. Effects of silymarin on the expression of PCNA, which is an indicator for cell proliferation in regenerating rat liver after 70% partial hepatectomy (PH) was determined by microscopy, RT-PCR and western blotting. The results indicated that silymarin could stimulate PCNA expression, presumably promote DNA synthesis in the nucleus and accelerate the hepatocyte proliferation in regenerating rat liver induced by PH.

REFERENCES


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