Inductive Effects of Ginseng Saponins on the Rat LDH A-gene and the Synthetic rate of Hepatocyte DNA in Regenerating Rat Liver Cells

Kye Jin Yoo, Kwang Youl Lee and Seung Ki Lee
College of Pharmacy, Seoul National University, Seoul 151-742, Korea

Abstract□ The effects of ginseng saponins, G-Rb1 and G-Rc on the rat liver LDH A-gene transcriptional activity was investigated during pre-replicative phase of rat liver after partial hepatectomy. Changes in LDH A-mRNA levels in regenerating rat liver after intraperitoneal administrations of G-Rb1 or G-Rc were tested by slot blot hybridization methods. The results showed that G-Rb1 (1 mg/100g B.W) and G-Rc (1 mg/100g B.W) caused marked increases of LDH A-mRNA contents by respectively 1.9- and 1.5-fold in rat liver at 5-hours after partial hepatectomy. Dose dependent effect of G-Rb1 and G-Rc (1-25 mg/100g B.W) on the LDH A-mRNA levels on regenerating rat liver were also analyzed. The maximal increases of liver LDH A-mRNA levels were observed with the doses of 1 mg for G-Rb1 and 5 mg for G-Rc. However, when the administration doses of G-Rb1 and G-Rc were increased to 20 mg, G-Rb1 caused a marked decrease of LDH A-mRNA level to 61% of those in sham-operated rat liver. In contrast, G-Rc slightly decreased the liver LDH A-mRNA contents by 30% as compared to those of the maximum value but still maintained 22% higher LDH A-mRNA levels then those of sham-operated rate liver.

On the basis of these experimental results, we conclude that ginseng saponin, G-Rb1 and G-Rc have stimulatory effect at the lower concentration (1 mg/100g B.W) and inhibitory effect at the higher concentration (20 mg/100g B.W) on the LDH A-gene transcription during regeneration of rat liver.

Additionally we also investigated the stimulatory effects of ginsenosides on the protein and DNA synthetic activities in hepatocyte primary cell cultures isolated from regenerating rat liver. Both of G-Rc and -Re increased the synthetic rates of hepatocytes proteins and DNA at the administration doses of 50 ug and 100 ug/3 ml/dish respectively representing 1.3-1.6 fold increases. From these results we postulate that G-Rc and -Re may have a mitogen enhancer activity for the hepatocyte proliferation during rat liver regeneration period.

Keywords□ Inductive effects of ginsenosides, G-Rb, -Rc, and -Re, rat LDH A-gene transcription, the synthetic rate of proteins and DNA in regeneration rat liver.

Introduction

For the last two decades or so many experimental evidences have been reported showing that the synthesis of RNA in rat liver can be stimulated by a single intraperitoneal injection of ginseng saponin extract of *Panax ginseng* C. A. Meyer[1-4]. The enzyme activity of RNA polymerase I and II was reported to be activated or inhibited by intraperitoneal injection of purified ginsenosides Rb1 and -Rc respectively[5,6]. It is not clear however whether or not the effect of ginsenosides on the enzyme activity is due to modulation of the enzyme itself or to the increased synthesis of the enzymes. Other investigations have also reported that ginseng saponins can increase the synthetic rate of serum proteins such as albumin and gamma-globulin in rat liver[7,8]. Furthermore, ginsenosides administration was shown to increase the activity of the glycolytic enzymes such as pyruvate kinase, phosphohexose isomerase, and lactate dehydrogenase[9-11].

All of these experimental evidences clearly indicate that ginseng saponins play a regulatory role on the hepatocyte gene expression. It is not clear

however what the molecular action mechanism is by which the expression of hepatocyte genes can be positively regulated in response to ginseng saponin treatment.

However several laboratories presented experimental evidences suggesting that some biochemical actions of ginsenosides may be mediated by cAMP as a second messenger. For example, Seo *et al.*[12,13] have demonstrated that ginsenosides Rb1 and -Rc exerted reciprocal effects on adenylate cyclase and guanylate cyclase. Nikaido *et al.* [14] demonstrated that ginsenosides present in the roots of *Panax ginseng* C.A. Meyer had an inhibitory effects on cAMP phosphodiesterase. Furthermore, Jin *et al.*[15] reported an experimental evidence suggesting that ginseng saponins may have the effect of stimulation on cytosolic cAMP synthesis, and both diol saponin and triol saponin may have an effect on pepsinogen secretion in isolated rabbit gastric glands. With all these experimental results in mind, we aimed in this study to quantitatively test the inductive effect of three purified ginseng saponins (G-Rb1, -Rc, and -Re) on a specific gene expression such as lactate dehydrogenase A-gene in regenerating rat liver. Additionally, we also aimed to systematically examine the effect of ginsenosides on the protein synthetic activity and DNA synthetic activity in the rat liver primary cell cultures isolated from the partially hepatectomized rat liver.

A single intraperitoneal injection of purified G-Rb1, and -Rc into partially hepatectomized rat was found to increase the rate of LDH A-mRNA synthesis in liver cells by the time-dependent and the dose-dependent manners, of which assays were determined by dot blotting hybridization method using ^{32}P-labelled LDH A-cDNA as a probe. Additionally we also observed in the primary hepatocyte cultures that the rates of protein and DNA synthesis were accelerated by G-Rc and -Re treatments.

From these results we conclude that ginseng saponins, G-Rb1, -Rc and -Re have inductive effects on the syntheses of proteins and DNA in regenerating rat liver. Furthermore we propose a hypothesis suggesting that ginsenosides G-Rc and -Re may play a role as an mitogen enhancer during regeneration period of rat liver.

Materials and Methods

Animals

Male SD rats weighing about 200 g were used for these experiments. These animals were supplied by SNU Animal Supply. Three or four rats were used for each experimental group. Partial hepatectomy by which two-thirds of the rat liver were excised was performed by the procedure of Higgins and Anderson[16], and sham operations were performed similarly except for the removal of the lobes. The animals were fed only water after the surgery. Ginsenosides dissolved in saline solution were injected intraperitoneally at one hour before partial hepatectomy.

Materials

DL-[4,5-^{3}H] Leucine (45 Ci/mmol) and [methyl-^{3}H]-TTP (30 Ci/mmol) were purchased from Amershm. Waymouth MB752 cell culture medium was obtained from GIBCO. Collagenase type I (125 u/mg) was obtained from Sigma. Nitrocellulose papers were obtained from Schleicher & Schuell. Guanidinium isothiocyanate and enzymes were obtained from BRL. N^6, O^2-dibutyryl cAMP was purchased from Boehringer Manheim. D.L-propranolol and other Chemicals were purchased from Sigma.

Extraction and purification of ginseng saponins

Roots of *Panax ginseng* C.A. Meyer produced in Kumsan, Korea were powdered and extracted with 0.05 M Tris-HCl buffer (pH 7.6) under stirring for 48 hrs in a cold room. Purification procedures for ginseng saponins were carried out by the method of Shibata as previously described[17].

Isolation and purification of poly(A) mRNA

For the preparation of RNA, livers were removed and immediately homogenized in 4 M Guanidinium isothiocyanate using a polytron homogenizer and total poly(A) mRNA was isolated by the methods of Chomczynski[18].

Determination of LDH A-mRNA concentration in rat liver

After glyoxylation, poly(A) mRNA was blotted on nitrocellulose paper. Prehybridization and hybridization with ^{32}P-labelled LDH A-cDNA were performed as described by Thomas[19], and then the filter was washed 5 times with 2x SSC containing 0.1% SDS at the room temperature and 2 times with 0.1 × SSC containing 0.1% SDS at 50 °C for 20 min. Autoradiography was performed with X-ray film (Kodak, X-Omat, AR) for 3 days exposure at −70 °C. Densities of autoradiogram were determined by scanning with a densitometer.

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from hepatectomized rat liver by using a two step perfusion based on the method of Dickins and Peterson[19]. The rat was anesthetized with urethane (1 g/kg, i.p.). The abdomen was showed, disinfected with Iodine Tincture, and strict aseptic technique was maintained throughout all procecure. The viability of the final cell culture was assessed by the addition of 1 m*l* of 0.4% Trypane Blue in 0.9% NaCl and mixing for 5 min. The visable cells were examined by a microscopy.

Assay of protein and DNA synthetic activity

The protein synthetic activity was determined by radiolabelling[20]. Culture medium was aspirated off and fresh medium containing [^{3}H]-Leucine (1uCi/m*l*) was added to the culture. After 20 hrs later, the medium was removed and cells were scrapped into a test tubes containing same volume of 20% TCA. The cells were pelleted by centrifugation at 2000 × g for 10 min, and washed twice with 10% TCA. The final pellet was digested in 0.5 m*l* of 0.5 N-NaOH and the radioactivity incorporated was counted. The DNA synthetic activity was determined by measuring the incorporation of [^{3}H]-Thymidine (1 uCi/m*l*. The method was similar to that of protein synthesis

Results and Discussion

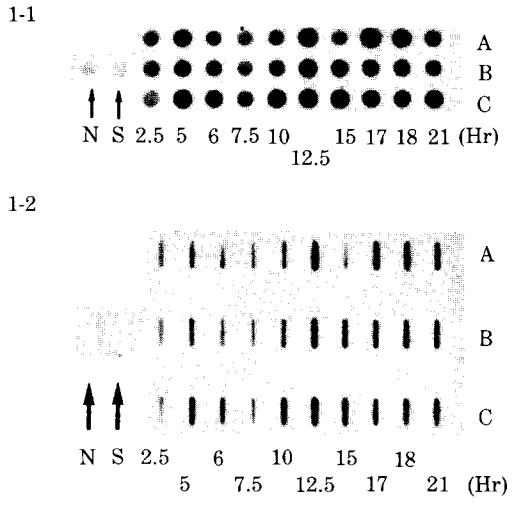


Fig. 1. Time course effects of G-Rb1 and G-Rc on the LDH-A mRNA concentration during the regenerating rat liver by Dot Blot and Slot Blot Analysis. Livers of three rats were used for each RNA preparation. Each ginsenoside and saline as a control were intraperitoneally injected to rats 1 hours before partial hepatectomy.
At each time point after pH, decapitation was performed and then regenerating liver was obtained. Total RNA preparation was done by AGPG method and then poly (A) mRNA was prepared.

Fig. 1-1 Dot Blot Analysis: 3.2 g of poly (A) mRNA was each spotted onto nitrocellulose filter. Hybridization was performed with LDH-A cDNA (1600) prepared by Nick translation

Fig. 1-2 Slot Blot Analysis: 1.6 μg of Poly (A) mRNA was each spotted onto NC filter. A is G-Rb1 treated, B, saline treated, and C, G-Rc treated. N is normal and S, sham-operated.

Effect of ginseng saponin G-Rb1 and G-Rc on the concentration of LDH A-mRNA in regenerating rat liver

In previous report[21], we have determined the transcriptional rat of LDH A-gene which was found to increase during the pre-replicative phase of regenerating rat liver and suggested that the induction mechanism of LDH A-gene transcription could be mediated by cAMP via β-receptor. In this study, the synergistic effect of ginseng saponins on the induction of LDH A-gene transcription was examined during regeneration period of rat liver. A

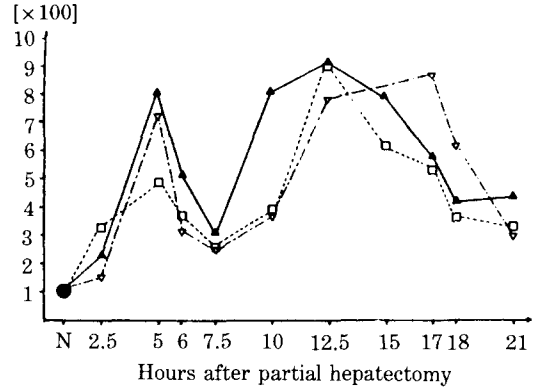
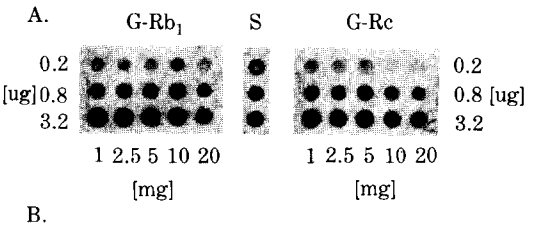


Fig. 2. The relative densitometric scanning profile of Fig. 1.
N: LDH-A mRNA of normal liver
□: LDH-A mRNA of saline-treated liver
▲: LDH-A mRNA of G-Rb1-treated liver
▽: LDH-A mRNA of G-Rc-treated liver



B.

S ↓	1 mg	2.5 mg	5 mg	10 mg	20 mg	← D
100	194	157	148	95	61	G-Rb1
	155	132	171	129	122	G-Rc

Fig. 3. Dose effects of G-Rb1 and G-Rc on the LDH-A mRNA concentration.
A: Dot blot analysis G-Rb1 and G-Rc were each injected to rats intraperitoneally with graded doses (1 mg, 2.5 mg, 5 mg, 10 mg, and 20 mg/100g body weight) at 1 hours before partial hepatectomy. At 5 hour after pH, liver was removed and poly (A) mRNAs prepared. Poly (A) mRNA were analyzed by Dot Blot Hybridization method.
B: The relative amount of LDH-A mRNA by densitometric scanning analysis of A.S is saline-treated.

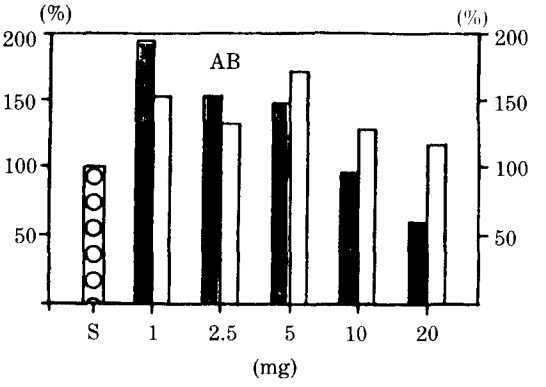


Fig. 4. The relative densitometric scanning profile of Fig. 3.
A: LDH-A mRNA of G-Rb1 treated
B: LDH-A mRNA of G-Rc treated
S: LDH-A mRNA of saline treated

single intraperitoneal injection of G-Rb1 or G-Rc (5 mg/100g B.W) dissolved in saline solution into rat time-dependently stimulated the LDH A-mRNA synthesis in regenerating rat liver (Fig. 1). The relative concentrations of LDH A-mRNA induced by the administration of ginsenosides were plotted as a function of time after partial hepatectomy (Fig. 2). Biphasic induction of LDH A-mRNA synthesis was observed during pre-replicative phase of regenerating rat liver as previously reported[21]. Intraperitoneal injection of G-Rb1 or G-Rc into rat showed also biphasic increases of LDH A-mRNA concentration. However, the ginsenosides even further enhanced the level of LDH A-mRNA by 1.5-1.7-fold at the first surge time as compared to that of regenerating rat liver and suggested that the in-regenerating rat liver. The ginsenosides though did not change the maximum concentrations of LDH A-mRNA at the second surge period but seemed to alter the maximum induction time period. The action mechanism for the synergistic effect of G-Rb1, and G-Rc on the LDH A-gene transcription is not well understood. However, previous reports[12,14] demonstrated that ginsenosides exert its stimulatory effect on adenylate cyclase activity and/or its inhibitory effect on cAMP-phosphodiesterase activity. Additionally the synergistic effect of ginsenosides on the induction of LDH A-gene transcription was temporally well matched with those of the induction time of cytosolic cAMP concentration after partial hepatectomy. Therefore it is anticipated that the action mechanism of ginsenosides may be mediated by cAMP as a second messenger al-

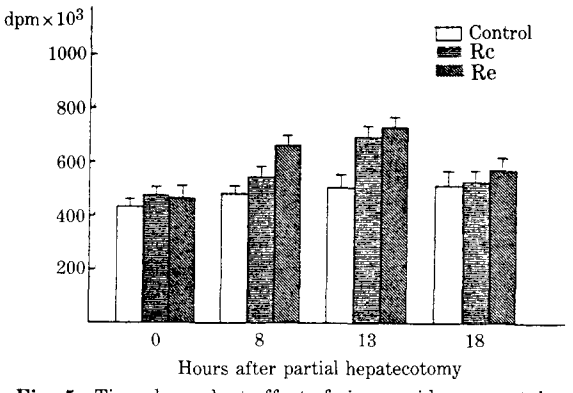


Fig. 5. Time-dependent effect of ginsenosides on protein syntheses in primary culture of regenerating rat hepatocytes

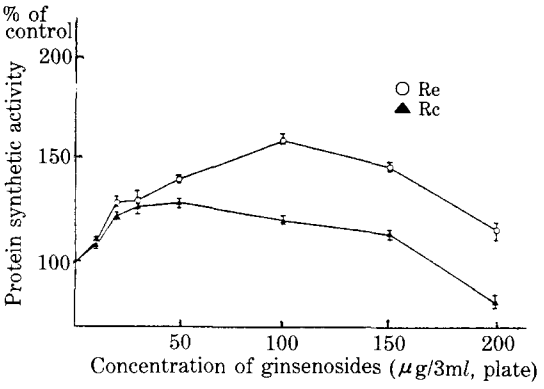


Fig. 6. Protein synthetic activities in regenerating rat liver were measured at 13 hr after pH

though this notion is remained to be clarified.

Dose-dependent effect of G-Rb1 and G-Rc on the induction of LDH A-gene transcription

After a single intraperitoneal injection of G-Rb1, or G-Rc, with the indicated administration doses in the range of 1 mg-20 mg/100g body weight, the effect of the ginsenosides on the concentration of LDH A-mRNA was examined by dot blotting hybridization method (Fig. 3). The relative concentrations of LDH A-mRNA in response to the ginsenosides administrations are shown in Fig. 4. As can be seen from Fig. 4, the maximum induction effect of G-Rb1, (2-fold-) or G-Rc (1.7-fold increase) was observed at 1 mg or 5 mg/100g b.wt respectively. As the administration doses of G-Rb1 were increased to 10-20 mg/100g b.w, the concentration of LDH A-mRNA were rather decreased to the lower level of control. The levels of LDH A-mRNA were also decreased at the higher dose of G-Rc than 10 mg/100g b.w. but maintained higher level than that of control. The inhibitory effect of G-Rb1 and G-Rc at the high doses is not well understood but very interesting phenomenon to be explored.

The stimulatory effects of ginsenosides G-Rc and -Re on the total protein and DNA synthetic activities

On the basis of previous reports suggesting that total saponin extract of *Panax ginseng* can stimulate the total protein and DNA synthetic activities, we systematically investigated the effects of purified ginsenosides, G-Rc and -Re utilizing the rat liver primary cell cultures isolated from the hepatectomized rat liver. The effects of ginsenosides, G-Rc and -Re on the synthetic rates of protein and DNA were determined by measuring the incorporation rates of [^{3}H]-Leucine and [^{3}H]-Thymidine into hepatocyte proteins and DNA respectively. As shown in Fig. 5, both of G-Rc and -Re had stimulatory effect on the protein synthesis with the peak values (1.3- and 1.6-fold respective increases) in the hepatocyte primary cell cultures isolated from regenerating rat liver at 13 hrs after PH. This result also shows that G-Rc and -Re had no significant stimulatory effect on the protein synthesis in the hepatocyte cell cultures isolated from normal and regenerating rat liver at 18 hrs after PH. This result suggests that the ginsenosides may not be able by itself to induce the initiation of protein synthesis but exert its effect synergistically on the protein synthetic rate in the cells which were already triggered for the initiation of protein synthesis. The dose-dependent effects of G-Rc and -Re on the protein synthetic rate were also determined in the range of 10-200 ug/3 ml/dish of the ginsenosides (Fig. 6). As can be seen in Fig. 6, the maximum inductive effect of G-Rc and -Re on the protein synthesis was observed respectively at the doses of 50 ug and 100 ug/3 ml/dish. At the higher administration doses than 200 ug/3 ml/dish of the ginsenosides, G-Re had no

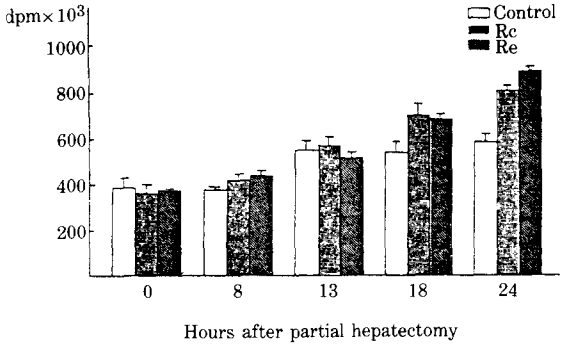


FIg. 7. Time-dependent effect of ginsenosides on DNA syntheses in primary culture of regenerating rat hepatocytes

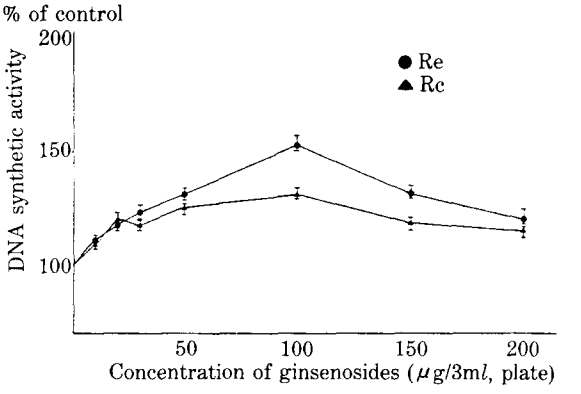


Fig. 8. DNA synthetic activities in regenerating rat liver were measured at 18 hrs after pH

significant stimulatory effect on the synthetic activity but G-Rc had rather repressive effect on the protein synthetic activity.

By using Trypane Blue exclusion assay, the hepatotoxic effect induced by G-Rc and -Re was examined at the high administration doses (200 ug/3 ml/dish) with which administration dose of ginsenosides the synthetic activity of hepatocyte proteins was repressed (The data is not shown). The result indicates that the viability of the hepatocytes was not significantly altered at the indicated administration doses of G-Rc and -Re. Therefore it is conceivable that the inhibitory effect of high doses of G-Rc and -Re on the hepatocyte synthetic activity is not due to the hepatotoxic effect of the ginsenosides.

The time-dependent induction effects of G-Rc and -Re on the synthetic activity of hepatocyte DNA were also determined as shown in Fig. 7. This result showed that the stimulatory effect of the ginsenosides was not apparent in the hepatocyte cultures isolated from hepatectomized rat liver at 13 hrs after partial hepatectomy but was statistically significant in the cell cultures isolated from rat liver after 18 hrs post-hepatectomy. This phenomenon again supports for the previous notion suggesting that the induction mechanism of ginsenosides for the protein synthesis may not be directly enhancing the initiation rate but rather synergistically exerting its effect on the protein synthetic activity in the cells previously triggered for the protein synthesis. The dose-dependent induction effect of G-Rc and -Re on the DNA synthetic activity was also determined in the hepatocyte cultures (Fig. 8). The maximum stimulatory effect of G-Rc and -Re on the hepatocyte DNA synthesis was observed at the administration dose of 100 ug/3 ml/dish representing respectively 1.3- and 1.6-fold increases. From these results we conclude that G-Rb1, -Rc, and -Re have inductive effects on the LDH A-gene transcription, protein and DNA synthesis in regenerating rat liver. Additionally on the basis of the result as described we propose a notion suggesting that G-Rc and -Re may play a role as a mitogen-enhancer. However further investigation needs to be done to elucidate the action mechanism of ginsenosides for the inductive effects on hepatocyte protein and DNA synthetic activities.

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