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Effects of Ginseng Saponin on DNA Strand Breaks and Replication Inhibition by Benzo(a)Pyrene in CHO-K1 Cells

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Abstract□The effect of saponin extracted from *Panax ginseng* C.A. Meyer on DNA repair and replicative DNA synthesis were examined in CHO-K1 cells cotreated with benzo(a)pyrene and rat liver S-15 fraction. The DNA strand breaks inititated by benzo(a)pyrene metabolites were measured by alkaline elution technique. The addition of ginseng saponin to the culture media resulted in decrease of benzo(a)pyrene-induced DNA strand breaks, and restored the suppressed-semiconservative-DNA-synthesis by the carcinogen. DNA repair synthesis in the damaged cells was also elevated by the ginseng treatment when the repairing activites were measured for the [^{3}H]-thymidine incorporation into the carcinogen damaged cellular DNA. Comparative analysis of DNA-adducts of benzo(a)pyrene metabolites in microsomes suggested that ginseng saponin treatment in rats reduced the formation of electrophilic metabolites of benzo (a)-pyrene in the rat liver microsomes.

Key words□ginseng saponin, benzo(a)pyrene, DNA strand breaks, DNA repair synthesis, metabolic activation, DNA adduct formation

Introduction

Ginseng has been widely used for centuries in many Asian countries as a tonic and prophylactic agent.[1] In recent years the prevention of cancer by ginseng treatment has been suggested as one of potential choice for the development of pulmonary adema induced by urethan and aflatoxin B_1.[2] Yun *et al.*[3] reported that natural killer cell activity depressed by carcinogen treatment such as urethan or benzo(a)pyrene (BP) was recovered to the level of controls by administration of ginseng.[3] The alteration of the metabolite patterns of BP and its reactive metabolites in rats were revealed as one of the pharmacological effects of ginseng.[4] Other supporting evidences for the ginseng as a potential theraputic agent for cancer are the enhancement of nuclear RNA polymerase activity, nuclear RNA synthesis, cytoplasmic heavy polyribosome content, amino acid incorporation into rat liver microsomes, and serum proteins.[5,6]

The initial step in the biotransformation of BP is catalyzed by mixed function oxidase system and ultimately epoxides are formed. BP-epoxides are spontaneously hydrolyzed to their dihydrodiols by epoxide hydrolase,[7] or covalent bind to tissue macromolecules such as proteins or nucleic acids.[8] BP-epoxides, particularly (+)-BP-anti 7,8-dihydrodiol 9,10-oxide (BPDE) have been shown to covalently bind to tissue macromolecules including DNA and this interaction with BPDE is thus far believed to be the most carcinogenic among the metabolic stero isomers of BP. BPDE modifed DNA almost exclusively at the position of C2 exocyclic amino group of deoxyguanosine (dGua).[9] The BPDE-DNA adduct formation is a critical molecular alteration since the persistence of this adduct during DNA replication leads to mutation, the initial step for transformation and tumorigenesis.[10] It has also been known that the ultimate carcinogenic form of BP blocks DNA replication in mammalian cells by forming a block in the intiation of replication at lower concentrations and in DNA elongation at higher concentrations.[11]

In this paper we investigated the effect of saponin fraction from *Panax ginseng* C.A. Meyer on DNA replication and DNA repair synthesis using the pH step alkaline elution technique. It is our understanding that saponins reduce the formation of DNA-BP metabolite adduct and have a relationship with the reduced inhibition of DNA-synthesis initiation by BP in CHO-K1 cells in the presence of metabolic activation system.

Materials and Methods

1. Animals

Male Sprague-Dawley rats of 180 g or male ICR mice of 20 g at the time of experiments were bred and maintained in KGTRI Animal Laboratory (Taejeon, Korea). The animals were housed in rooms designed to maintain 22~24℃ with 12 h light/dark cycle, 50% humidity. Food and water were fed ad libitum

2. Chemicals

7,8-Dihydroxy-7,8-dihydro benzo(a)pyrene was purchased from Midwest Institute, Kansas City Mo. Other reagents enzymes and solvents were all obtained from Sigma Chemical Co., St. Louis, Missouri or Aldrich Chemical, Milwaukee, Wisconsin. The Labeled compounds were all purchased from Amersham International LTD., Amersham.

3. Cell culture

Chinese hamster ovary (CHO-K1) cells were exclusively used throughout this investigation. Monolayer cultures of this cell line were grown at 37℃ in humidified 5% CO_2 and the medium used was Eagle's minimum essential medium (MEM) supplemented with 10% calf serum, penicillin G (100 units/m*l*) and streptomycin (100 μg/m*l*).

4. Measurement of DNA strand breaks

For the determination of DNA single strand breaks, CHO-K1 cells were prelabeled with 0.2 μCi/m*l* for 12~24 h in the medium containing[^{14}C]-thymidine (Amesham, 50~60Ci/mmol). After labeling the radioactive medium was discarded and the cells were washed with PBS, chased in the growth medium for 4 h and then treated with BP. Either panaxadiol or panaxatriol saponin was treated for 1 h with BP if necessary. The cells were detached by gentle scraping with a rubber policeman and then dispersed in cold PBS solution. Alkaline elution was performed according to Kohn *et al*[12] with minor modifications.

5. DNA replication inhibition test

CHO-K1 cells ($1.0\sim5.0\times10^5$) were prelabeled with [^{14}C]-thymidine (0.01 μCi/m*l*, 52 mCi/mM) for more than 24 h, washed and exposed to BP or BP with total saponin (10 μg/m*l* in saline) for 30 minutes. At various time after treatment, the cultures were pulse labeled with [^{3}H]-thymidine at a final concentration of 5 μCi/m*l* for 10 minutes. The cells were quickly rinsed in ice-cold saline citrate and scrapped off and collected on Whatman GF/C papers. The radioactivities of [^{3}H] to [^{14}C] was determined.

6. Binding of BP metabolites to DNA

Male Sprague Dawley rats of 200 g or ICR mice of 20 g were dosed with total saponin (200 mg/kg b.w.) by intraperitoneal injection for 3 consecutive days. Control animals were given saline by p.o. After 16 h fasting from the last administration of saponin the animals were killed by cirvical dislocation and livers removed for the microsomal preparation. The reaction mixture contained 20 μM of [^{3}H]-BP (14.5 μCi/nmol), 1 mg of calf thymus DNA, 0.75 mg of microsomal protein and cofactors (0.7 mM NADPH, 10 mM glucose-6-phosphate, 0.35 U/m*l* of glucose-6-phosphate dehydrogenase, 0.1 mM EDTA, and 5 mM $MgCl_2$) in 0.1 M potassium phosphate buffer, pH 7.5. After 60 min incubation, the DNA was isolated from the incubation mixtures[13] and subjected to enzymatic hydrolysis at 37℃ and then proteins and unbounded metabolites were eliminated Nucleosides covalently bound to BP-metabolites were isolated from elution of a SEP-PAK C18 catridge (Waters, Co.) as a precolumn and then applied μBondapak C18 HPLC column (25×0.46 cm, id), developed with 30 min linear gradient of 50~80% methanol followed by 90% methanol for 15 min (0.8 m*l*/min, 10 drops/fraction). The elluent was monitored at 254 nm and the fractions were collected and used to determine radioactivity. The gradient of 45~85% methanol was given in the experiment of LH-20 column (1.5×18 cm).

7. GC analysis of BP-metabolite-deoxyribonucleo-

side adducts

3.3 μmole of BP-7,8-diol was incubated with MC (20 mg/kg b.w. for 3 consecutive days)-induced rat liver microsoems (400 μg protein) in the presence of dGua. The incubation mixture (final volume, 0.5 m*l*) contained 10 mM tris-HCl(pH 7.9), 0.1 M NaCl, 1 mM $MgCl_2$ and dGua (0.3 mg/m*l*). The separation of modified nucleosides was performed by Sepak C_{18} catrige using water and methanol, sililated with BSTFA[N, O-Bis(trimethylsilyl)-trifluoro-acetamide] and then applied to GC. GC condition: column; SPB-1 fused silica capillary (025 mm i.d.×30 m, 1.0 μm, Supelco), Temperature; 230℃ to 300℃, Carrier; 1.7 m*l*/min, 5% CH_4/Ar (split ratio=20 : 1), Detector; ECD (^{63}Ni), GC model; Hewlett-Packard 5840A.

8. Enzyme assays

Glutathion-S-transferase (GSH-T) activity in cytosolic fraction was measured by the method of Habig *et al.*[4], employing 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Glucose-6-phosphate dehydrogenase (G6PDH) activity[15] and protein[16] were also determined as described previously. Microsomes[17] and ginseng saponins[18] were prepared as described previously.

Results

Alkaline elution assessed DNA scission on the basis of an increase in the rate of elution of single stranded DNA fragment as a function of the number of DNA breaks such as nicks or gaps. Ginseng saponins inhibited the accumulation of single strand breaks by 10 μM BP using rat S-15 fraction as a metabolic activation system (Fig 1.). When CHO-K1 cells were cotreated with BP and S-15 microsomal fractions in the presence of 10 μg/m*l* of ginseng saponins, the radioactivity of remaining on the filter paper during 12 h of elution period was higher than that by treatment of BP and S-15 fraction. There were no significant differences between the diol (Fig. 1A) and triol (Fig. 1B) saponins in their effects. These results indicate that ginseng saponin treatment inhibited formation of the DNA breaks in carcinogen damaged CHO-K1 cells.

Fig. 2 represents the recovery profile of DNA synthesis from the inhibition by the activated BP. It has been known that DNA damage by chemicals or physical agents DNA sysnthesis either by inhibiting the initiation of replication or by blocking the progress of replication fork. The inhibition of DNA sysnthesis by the activation of BP in mammalian cells has been well described.[11] The post treatment of 10 μg/m*l* ginseng total saponin restored the DNA synthesis in 1 hr, while control CHO-K1 cells treated with BP and S-15 fraction followed by allowing to recover in the regular medium were required

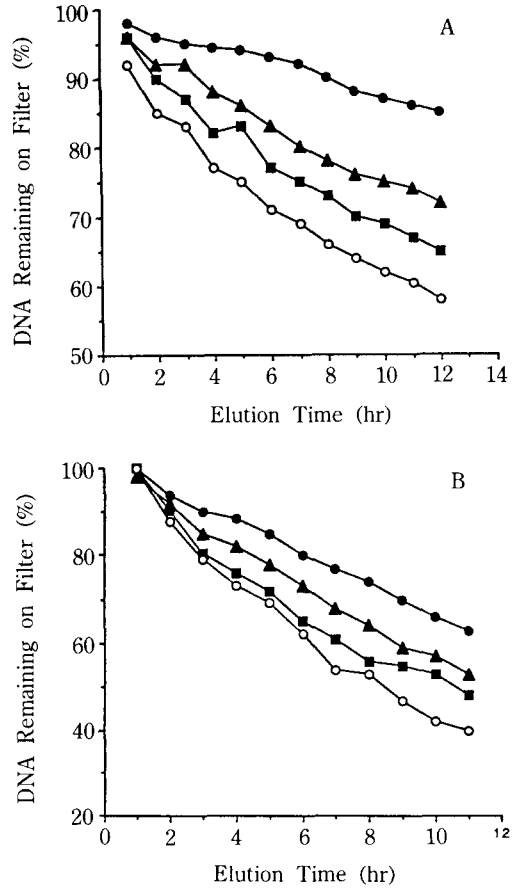

Fig. 1. Effects of panaxadiol saponin (A) and panaxatriol saponin (B) on DNA single strand breaks induced by benzo(a)pyrene in the presence of S-15 fraction ●; control, ▲; 10^{-5} M BP+S-15 fraction+saponin (10 μg/m*l*), ■; 10^{-5} M BP+S-15 fraction+saponin(1 μg/m*l*), ○; 10^{-5} M BP+S-15 fraction.

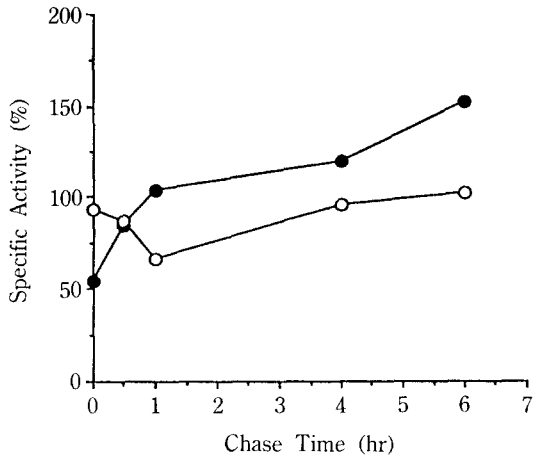

Fig. 2. Effects of total saponin on the recovery of DNA synthesis inhibition induced by benzo(a)pyrene in the presence of S-15 fraction with the lapse of time. ○; control, ●; Total saponin treatment (10 μg/ml)

4 h to recover the DNA synthesis activity. The restoration effect of total saponin on the inhibition of DNA synthesis by BP was observed significantly in the range from 0.1 to 10 μg/ml (data not shown).

To determine whether the increased rejoining of single strand and [^{3}H]-thymidine incorporation into cells by ginseng total saponin treatment were caused by the inhibitory effects of ginseng saponins on the microsomal activation system, the effects of saponin on the formation of DNA-BP adducts were examined. In the positive control experiment, 3-methylcholanthrene(MC)-induced microsomes were used as an activation system including 7,8-diol-BP as a standard substrate. The identification of the secondary metabolite-dGua adduct derived from metabolites of BP-7,8-diol was attempted by using gas chromatographic technique with electron capture detector (ECD).

The binding of dGua adduct with the metabolites of 7,8-diol-BP was shown as two peaks seperated (26.9 and 27.6 min) in the chromatographic profiles (Fig. 3C). The major adduct detected in BP-treated rodent[19] and human cell cultures[20] is the (+) anti-BPDE : dGua adduct,[21] but the gas chromatographic analysis was attempted for the first time in order

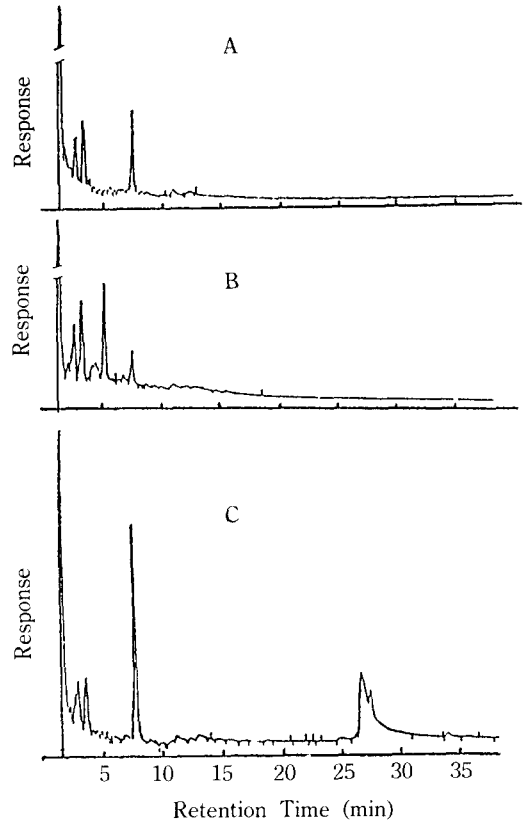

Fig. 3. Comparison of benzo(a)pyrene-metabolite-deoxynucleoside adducts formation in control (A), total saponin treatment (B), BP-7,8-diol metabolite-dGua adduct formation (C). The relative area percentages of 7.9 min peaks were 6.559 (a), 3.863 (b), 16.094 (c) and those of 26.8 and 27.6 min peaks were 10.317, 9.623, respectively.

to detect the dGua adduct with the oxidation products of BP-7,8-diol in pmol level. These dGua adducts with the secondary BP-metaboites were not observed in control and saponin-treated rats (Fig. 3 A and B. On the other hand, as was shown in the HPLC elution profile of Fig. 4, the ability of cytochrome P-450-linked mixed function oxidase system to induce electrophilic metabolites of BP in mice was certainly depressed by the total saponin treatment. The final BP-metabolite-DNA adduct formation in rats was not so remarkable as that in mice. The elution profile of the final modified BP-deoxyribonucleoside adduct from LH-20 column revealed four different peaks (Fig. 5). These addu-

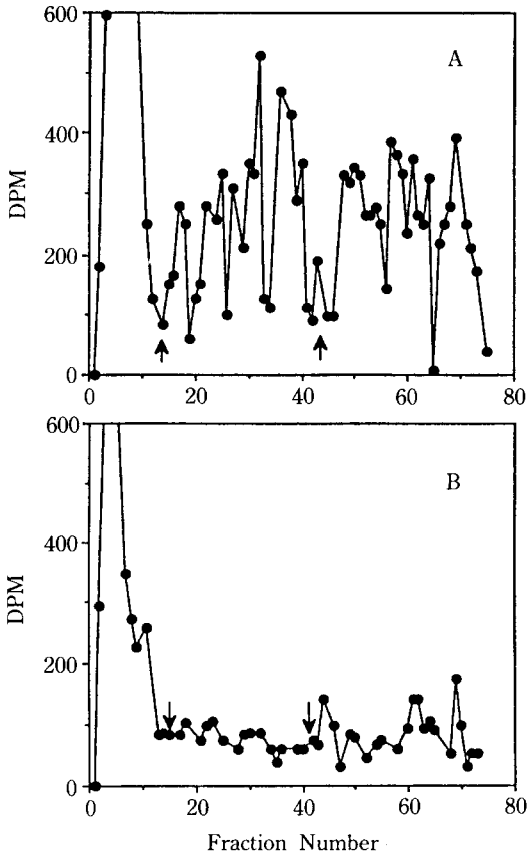

Fig. 4. Elution profile from HPLC of hydrolyzed calf thymus DNA previously incubated with [^{3}H] benzo(a)pyrene and liver microsomes from control (A) or saponin-treated ICR mouse (B), respectively.

cts have not been indentified, but the radioactivities in peaks 1,3 and 4 demonstrate that ginseng total saponin treatment reduced specific DNA-BP-metabolite adducts formations by 18.6, 18.3, 12.2%, respectively. An independent experiment was carried out to examine the effect of ginseng saponin on the cytosolic factors containing the microsomal activation system. As was shown in Table 1, the G6 PDH activity was reduced to 76% of control level by ginseng saponin treatment. But the GSH-T activity remained unaffected by the treatment.

Discussion

The present study suggests that ginseng saponin

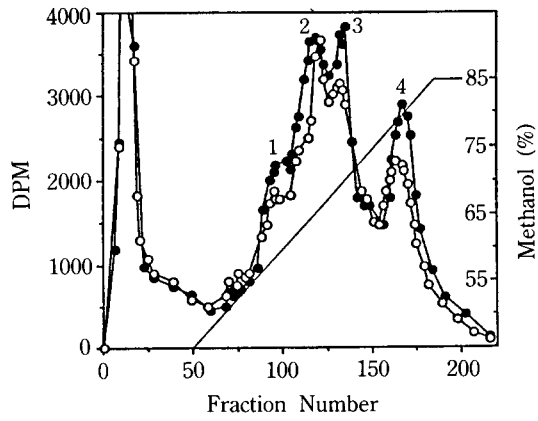

Fig. 5. Comparison of the elution profile of DNA-benzo (a)pyrene metabolite adducts from LH-20 column in control ● and saponin treated O rats.

inhibits the metabolic activation of BP which is absolutely required for the initiation of DNA repair synthesis and the inhibition of DNA synthesis. Panaxadiol saponins, panaxatriol saponins, and total saponins reduced DNA single strand breaks suggesting that ginseng saponin treatment increased either the number of active replicons or the rate of the DNA repair synthesis (Fig. 1). The results in Fig. 2 allow a conclusion for the positive effects of ginseng saponin on the initiation of replicative DNA synthesis in mamalian cells. The *in vitro* BP-DNA binding studies indicated that the effect of ginseng saponin on the inhibition of activated BP-metabolites formation was not direct one since the indirect addition of saponin to S-15 preparation did not exert significant inhibition (data not shown). Thus, the binding of BP-metabolites to DNA was investigated using the microsomes which were isolated from the saponin-treated rat liver.

When the total amounts of the enzyme hydrolysates to DNA were normally compared by HPLC, the elution profiles on HPLC showed that the adducts of oxygenated BP-metabolites were significantly decreased specially in mice by total saponin treatment (Fig. 4). When the total nucleoside-adducts were applied to the LH-20 column, however, the relative amounts among a series of BP-metabolite deoxyribonuleoside adducts formed by rat liver monoxygenase system were to some extent reduced

Table 1, Effect of saponin fraction on glutathione S-transferase (GSH-T) and glucose-6-phosphate dehydrogenase (G6PDH) activity

	Control	Saponin (20 mg/kg b.w.)
GSH-T (umole/min/mg protein)	0.454± 0.051 (100)	0.461± 0.043 (102)
G6PDH (unit/mg protein)	0.042± 0.024 (100)	0.033± 0.013 (76)*

Data are expressed as the mean± S.D. of 5~12 individual values per group (S.D. rats). Values in parenthesis represent % of control activity. Liver cytosols were used as enzyme sources. Total saponin fraction (20 mg/kg b.w.) was administered intraperitoneally for 3 consecutive days. (*$p<0.005$ vs. control)

by the total saponin treatment (Fig. 5). These results indicate that administration of ginseng total saponin to the rodents reduced the activation of the microsomal monooxygenase system at the optimized dosage of total saponin (20 mg/kg b.w.), though the species variation was in existance.

The other factors also may be involved with the ability of DNA adduct formation such as the extent of conjugation, the production of DNA adduct, the production of BP-7,8-diol and its subsequent epoxidation, NADPH generation[22] in cytosol could be a factor for reducing the metabolic activation of BP in rat liver. If G6PDH activity decreases in CHO-K1 cells, BP-7,8-diol production and its subsequent epoxdation will appear to be considerably reduced and it seemes be unlikely that metabolic conjugation of BP undergoes a major changes as compared to controls.[23] Table 1 shows that G6PDH activity was significantly inhibited by 25% in liver cytosols from rats treated with ginseng total saponin, while GSH-T activity remains hydrolase activity by about 40% in ICR mice[24] and induced directly morphological reverse transformation in cultured Morris hepatoma cells.[25] The increase of epoxide hydrolase activity without concurrent induction of arylhydrocarbon hydrolase activity in rat liver by the treatment of ginseng total water extract[4] also provides a good evidence for the results. In this study we attempted to evaluate the biochemical roles of ginseng associated with the metabolic activation of BP and repair of BP-induced DNA associated with the metabolic activation of BP and repair of BP-induced DNA damage in CHO-K1 cells. The role of ginseng saponins *in vivo* may have a significance in explaining the relationships between specific DNA damage and its restoration in cultured CHO-K1 cells. These studies are in progress.

요 약

쥐의 간 상등액(S-15 fraction)을 대사 활성제로 사용하여 CHO-K1 세포에서 benzo(a)pyrene에 의해 유발되는 DNA 단사절단 및 수선속도저하 등 DNA 손상에 미치는 인삼사포닌의 영향을 알칼리 유출법을 사용하여 조사하였다.

10^{-5}M의 benzo(a)pyrene과 사포닌 분획(10 μg)을 처리하면 DNA 단사절단 속도가 감소하는 경향을 보였으며 benzo(a)pyrene에 의한 DNA 합성저하의 회복속도는 benzo(a)pyrene 단독처리 대조군의 4시간에 비해 약 1시간으로 단축되었다. 한편, 쥐간 마이크로좀 분획에 의한 DNA-BP 대사물질 포합체 형성은 사포닌 투여(3일, 경구, 20 mg/kg b.w.)로 감소되었는데 이와같은 결과는 DNA단사절단 감소 및 절단된 DNA 수선속도 회복에 benzo(a)pyrene의 대사적 활성화 억제가 관련되어 있음을 시사한다.

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