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Chemopreventive Effect of Saponins Derived from Roots of *Platycodon grandiflorum* on 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone-Induced Lung Tumorigenesis in A/J Mice

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This study examined the chemopreventive effect of saponins that were isolated from the roots of *Platycodon grandiflorum* A. DC (Campanulaceae), Changkil saponins (CKS), against the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), -on lung tumorigenesis in A/J mice. The mice were treated with a single NNK dose (100 mg/kg b.w., i.p.). CKS (0.5, 1, 4 mg/kg body wt.) was administered orally daily for 3 days/week beginning 1 day after the NNK treatment and was maintained throughout the experiment. The administration of CKS suppressed the NNK-induced increase in the level of proliferating cell nuclear antigen, which are a marker of cell proliferation, in the lungs of the mice 4 weeks after the NNK injection. Twenty-five weeks after the NNK treatment, the mice were sacrificed and the number of surface lung tumors was measured. CKS significantly reduced the number of lung tumors induced by NNK in a dose dependent manner. These results suggest that CKS suppresses the development of lung tumors and has a chemopreventive effect against NNK-induced mouse lung tumorigenesis.

Key words: Platycodi radix, Saponins, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone, Lung tumorigenesis, A/J Mice

INTRODUCTION

Lung cancer is the largest cause of cancer death worldwide, with cigarette smoking being the most significant risk factor (Hecht and Hoffmann, 1988; Hoffmann *et al.*, 1996). This highlights the need for additional lung cancer prevention strategies, such as chemoprevention. Chemoprevention is defined as the administration of natural or synthetic compounds that inhibit, block or reverse the process of carcinogenesis (Wattenberg, 1985). Chemoprevention using naturally occurring or synthetic compounds to arrest or reverse the carcinogenic process is an extremely important lung cancer prevention strategy. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most important carcinogenic nitrosamine on account of its strong potential in inducing lung neoplasms in rodents and its exposure to humans via smoking (Hoffmann *et al.*, 1996). There is evidence supporting NNK as a likely etiologic agent for smoking-related lung cancer (Hecht and Hoffmann, 1988; Belinsky *et al.*, 1990, 1992). The induction of lung tumors by NNK in A/J mice is a well-established model for examining lung carcinogenesis (Hecht *et al.*, 1989; Belinsky *et al.*, 1992). Therefore, it is important to estimate the effect of a chemopreventive agent on NNK-induced lung tumorigenesis in animal models in order to establish a useful chemopreventive agent against tobacco-induced lung cancer in humans.

Vegetables and fruits are associated with a reduced risk of cancer, including lung cancer (Block *et al.*, 1992; Voorrips *et al.*, 2000). Various dietary and synthetic compounds have been shown to inhibit the development of lung tumors in NNK-treated rodents (Xu *et al.*, 1992; Hecht, 1996; Castonguay and Rioux, 1997; Voorrips *et al.*, 2000). Among them, some of these agents, such as carotenoids and flavonoids, exert their activity as potential antioxidants and can scavenge free radicals (Xu *et al.*, 1992; van

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Zandwijk, 1995; Voorrips *et al.*, 2000). N-acethylcysteine has been reported to have protective effects against the induction of neoplastic and preneoplastic lesions in animals (De Flora *et al.*, 1995). The protective activity of the N-acethylcysteine can be attributed to its function as an antioxidant and/or as a nucleophile capable of scavenging carcinogens (van Zandwijk, 1995).

Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae) (four years old) has been used as a food as well as in traditional oriental medicine to treat adult diseases, such as, bronchitis, asthma and pulmonary tuberculosis, hyperlipidemia, and inflammatory diseases, as well as being taken as a sedative, Its biological significance has previously been reviewed (Lee, 1973). Previous studies found that Changkil (CK), which is the aqueous extract made from the root of *P. grandiflorum* cultivated for more than twenty years (Lee, S.H., 1991, Patent on the method of cultivating the perennial balloonflower, Patent No. 045971, Korea), prevented hypercholesterolemia and hyperlipidemia (Kim *et al.*, 1995) and modulated the functions of macrophages (Choi *et al.*, 2001). Recently, it was shown that CK had protective effects against prevented the acetaminophen- and CCl_4-induced hepatotoxicity and the progress of hepatic fibrosis in rats (Lee *et al.*, 2001, 2004a; Lee and Jeong, 2002). In addition, it was also reported that CK and the saponin fraction (CKS) derived from CK have potent antioxidant effects, such as a superoxide radical scavenging activity generated via the xanthine and xanthine oxidase system, and a reduction in ROS production by *tert*-butyl hydroperoxide in hepatocytes (Lee and Jeong, 2002; Lee *et al.*, 2004b).

Even though CKS exhibits antioxidant activity, its efficacy as a chemopreventive agent in NNK-induced lung tumors in A/J mice has not been examined. This study investigated the chemopreventive effect of CKS on NNK-induced early pulmonary proliferation as a proliferating cell number antigen (PCNA)-labeling index in lung proliferative lesions and lung tumorigenesis in A/J mice. The results indicate that CKS significantly prevented NNK-induced mouse lung tumorigenesis in the A/J mice.

MATERIALS AND METHODS

Preparation of CKS

CK refers to the aqueous extract obtained from the twenty-two years old roots of *Platycodon grandiflorum*, which was supplied by Jang Saeng Doraji Co., Jinju, South Korea. The composition of the *P. grandiflorum* root was previously published (Kim *et al.*, 1995). The CK and CKS were prepared as described previously (Lee *et al.*, 2001, 2004b; Lee and Jeong, 2002). CK was subjected to column chromatography over amberlite XAD-2, Diaion MCI Gel HP20 or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain the CKS, which is the saponin fraction of CK, as described previously (Tada *et al.*, 1975).

Animals and treatment

Six week old female A/J strain mice were purchased from Dae Han Laboratory Animal Research and Co. (Daejeon, Korea). The animals were given access to Purina Rodent Chow and tap water *ad libitum*, maintained in a controlled environment at 21 ± 2°C and 50±5% relatively humidity with a 12 hr dark/light cycle, and acclimatized for at least I week prior to use. NNK (purity > 99%) was obtained from Chemysin Science Laboratories (Lenexa, KS, U.S.A.) and dissolved in sterilized saline, and the mice were given the NNK solution at a single dose of 100 mg/kg body weight via an i.p. injection. CKS was suspended in sterilized saline and administered orally to the mice daily for 3 days/week beginning from 1 day after the NNK treatment until the end of the experiment (week 0-4 or week 0-25). The animals were randomly divided into 6 groups, each consisting of 20 mice. Group 1 was the untreated control. Group 2 was given CKS (4 mg/kg). Groups 3-6 were given NNK. Group 3 was given NNK alone. Groups 4-6 were administrated 0.5, 1, or 4 mg CKS/kg for 4 or 25 weeks. The body weights were monitored each week. Four weeks after the NNK injection, four mice in each group were sacrificed in order to estimate the pulmonary proliferating cell nuclear antigen (PCNA) level, as a marker of cell proliferation (tumor promotion). Twenty-five weeks after the NNK injection, 16 mice from each group were sacrificed under ether anesthesia in order to confirm the development of the lung tumors.

The estimation of lung tumors

The lungs from the mice sacrificed 25 weeks after the NNK treatment were fixed with 10% neutralized formaldehyde, and the number of visible tumors on the surface of the lungs were counted. Two observers counted the number of tumors independently. The difference between the two observer counts was < 5%.

Immunoblot analysis of PCNA

The soluble protein fraction was prepared from a 10% lung homogenate in a lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 2 mM leupeptin and 5 mM sodium vanadate) by centrifugation at 4°C for 60 min at 100,000 × g. The protein concentration was measured using the Bradford method. Electrophoresis and immunoblotting against the antiproliferating cell nuclear antigen (PCNA) or â-actin antibody (Calbiochem, La Jolla, CA, U.S.A.) were performed as previously described (Higaki *et al.*, 1993).

The blots were developed using a chemiluminescence (ECL) detection system (Amersham, Heights, IL, U.S.A.). The PCNA level was used as a marker of cell proliferation during tumorigenesis (Said and Medina, 1995).

Statistical analysis

The Dunnett's t-test was used to determine the statistical significance of the differences on the tumor multiplicity and a P value <0.05 was considered significant.

RESULTS

General observation

All the animals remained healthy throughout the experimental period. In the NNK-induced lung tumorigenesis experiment, the body weights of the mice were measured weekly. When mice were sacrificed, the mean body weights in the various groups were similar (data not shown). The mice tolerated the CKS treatment well, supporting normal growth in the rats with no adverse effects (data not shown).

Inhibition of cell proliferation in lung tumors by CKS

In order to determine the effect of CKS on cell proliferation in NNK-induced lung tumorigenesis, the PCNA expression level, which is a marker of cell proliferation, was estimated using immunoblot analysis. Fig. 1 shows the effect of CKS on the PCNA level. The expression of PCNA in the NNK-treated group was higher than that in the control group, and the CKS administration significantly decreased the NNK-induced increase in PCNA expression in a concentration dependent manner.

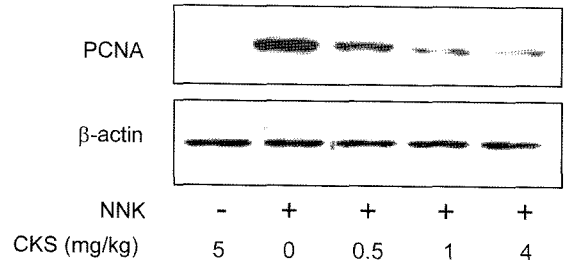

Fig. 1. Immunoblot analysis of NNK-induced PCNA expression . CKS (mg/kg) was administered to the mice 1 day after a single dose of NNK (100 mg/kg b.w., i.p.) until the end of the experiment as described in Materials and Methods. Four weeks after the NNK treatment, mice were sacrificed and the lung lysates (30 μg protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with an anti-PCNA or β-actin antibody.

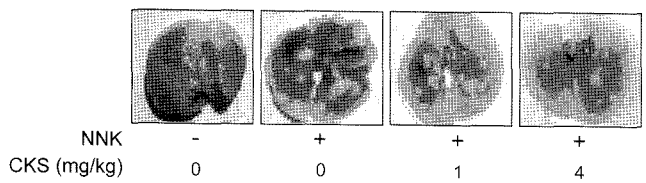

Fig. 2. Surface lung tumors induced by NNK were reduced by CKS. CKS (mg/kg) was administered to the mice 1 day after a single NNK dose (100 mg/kg b.w., i.p.) until the end of the experiment, as described in Materials and Methods. Twenty-five weeks after the NNK treatment, the mice were sacrificed and the lungs were fixed in a 10% neutralized formaldehyde solution.

Table I. Inhibition of NNK-induced lung tumors in A/J mice by CKS

Treatment group	Tumor incidence (%)	Tumor multiplicity[a]
Control	0 (0/16)	0
CKS 4	0 (0/16)	0
NNK	100 (16/16)	11.3 ± 1.2
NNK + CKS 0.5	100 (16/16)	9.4 ± 0.9 (17%)
NNK + CKS 1	100 (16/16)	6.8 ± 0.8[b] (40%)
NNK + CKS 4	100 (16/16)	3.1 ± 0.5[b] (73%)

CKS (mg/kg) was administered to the mice 1 day after a single NNK dose (100 mg/kg b.w., i.p.) until the end of the experiment, as described in Materials and Methods. Twenty-five weeks after the NNK treatment, the mice were sacrificed and the number of tumors on the lung surface was counted.

[a]Tumor multiplicity is the mean ± SE with percentage inhibition by CKS shown in parentheses.

[b]Significantly different from the NNK group ($P < 0.05$).

Inhibition of NNK-induced lung tumorigenesis by CKS

In the NNK-induced lung tumorigenesis experiment, the mice were administrated 0.5, 1, or 4 mg CKS/kg, beginning 1 day after the single NNK dose and continuing until the end of the experiment. As shown in Table I, at week 25, lung tumors were found in all of the NNK-treated mice in both the positive control and CKS-treated groups. However, lung tumors were not observed in the mice in the water- or CKS-negative control groups. Significant inhibition of tumor multiplicity was observed in the mice treated with CKS in a concentration dependent manner (Table I). The data showed that CKS reduced the number of the lung tumors induced by NNK (Fig. 2), and a ~40% reduction in the number of the lung tumors was observed in the mice administrated CKS at 1 mg/kg (Table I).

DISCUSSION

This study demonstrated that the administration of CKS decreased the lung tumor multiplicity in A/J mice (Table I). Therefore, these results provide strong evidence that CKS can inhibit NNK-induced pulmonary tumorigenesis in A/J mice. This inhibitory action of CKS may be enacted, at least in part, by inhibiting cell proliferation in the early

stages of carcinogenesis. NNK is activated by alkylating agents that attack the DNA, and cell division is needed to fix the mutations caused by the modified DNA in daughter cells. PCNA is a commonly used marker for proliferation and is a cofactor of DNA-polymerase. It is essential for DNA replication and DNA nucleotide excision repair (Hall *et al.*, 1990; Shivji *et al.*, 1992). The apparent increase in PCNA is a general feature of multi-step carcinogenesis. PCNA has been used to monitor the proliferation of tumors. The expression of PCNA is enhanced in proliferating tumor cells (Nguyen *et al.*, 2000). In this study, NNK exposure increased the level of cell proliferation activity in the lung and the lung tumors in the mice given NNK, and CKS had a lower PCNA-positive index in a dose-dependent manner than in the mice treated with NNK (Fig. 1). The inhibitory effect of CKS on NNK-induced lung tumorigenesis may be due in part to a modification of cell proliferation. These results suggest that CKS suppressed the development of the NNK-induced lung tumors, which was illustrated by the reduction in the number of the initiated cells. It was also demonstrated that CKS inhibited cell proliferation during the promotion phase of NNK-induced lung tumorigenesis in mice. This suggests that CKS can act as a useful chemopreventive agent against lung tumorigenesis. This is supported by the CKS suppressed NNK-induced increase in the PCNA level, which is a marker of cell proliferation and the development of lung tumors (Fig. 1, Table I). The number of mutation events may be reduced with the suppression of NNK-induced early proliferation by CKS. To our knowledge, this is the first demonstration of the anti-proliferative and anti-tumorigenic activities of CKS in the lung. The relative contribution by each component in the CKS mixture used in this study needs to be determined.

The mechanisms by which CKS exerts its inhibitory effect are not known. The K-*ras* gene is mutated in NNK-induced lung tumorigenesis, which might be a key factor that not only initiates but also promotes the development of lung tumors (Ronai *et al.*, 1993; Ziegel *et al.*, 2003). It was recently reported that superoxide production is related to the K-*ras*-driven oncogenic process, and that antioxidants may inhibit this process (Irani *et al.*, 1997). In a previous report, it was shown that CKS has potent antioxidant effects, such as reducing the superoxide radical scavenging activity and the level of ROS production by *tert*-butyl hydroperoxide (Lee *et al.*, 2004b). Therefore, CKS may suppress NNK-induced lung tumorigenesis via the inactivation of the K-*ras* gene by controlling the expression of the PCNA protein. This hypothesis is strongly supported by the recent finding that a K-*ras* gene mutation can enhance the lung adenocarcinoma cell proliferation (Okudela *et al.*, 2004). Whether or not this proposed mechanism is applicable to our model remains to be determined.

There is ample evidence suggesting that free radicals are involved in the carcinogenesis processes (Cerutti, 1994). It was reported that the mechanism of the inhibition of NNK-induced lung tumorigenesis by several compounds is due at least part to their antioxidant properties (Xu *et al.*, 1992; van Zandwijk, 1995; Voorrips *et al.*, 2000). As shown in this study, CKS has a protective effect against NNK-induced lung tumorigenesis in A/J mice. CKS has powerful antioxidant properties that are capable of scavenging the superoxide anion (Lee *et al.*, 2004b). Therefore, it is reasonable to assume that the inhibitory activity of CKS on NNK-induced lung tumorigenesis is due to its antioxidant activity. Treatment with NNK can increase the plasma levels of prostaglandin E_2 derived from cyclooxygenase-2, and cyclooxygenase-2 inhibitors, such as sulindac and NS-398, can lower the plasma levels of prostaglandin E_2, and suppress the NNK-induced lung tumorigenesis (Rioux and Castonguay, 1998; Castonguay and Rioux, 1997). Although the effect of CKS on the plasma levels of prostaglandin E_2 was not investigated in this study, the platycodin D contained in CKS inhibits prostaglandin E2 production in macrophages (Kim *et al.*, 2001). Furthermore, the aqueous extract from Platycodon grandiflorum induces apoptosis in human lung carcinoma cells (Park *et al.*, 2005). Therefore, the inhibitory effects of CKS may be due in part to the modification of cyclooxygenase-2 inhibition, the immune response, and the induction of apoptosis.

During the search for new cancer chemopreventive agents that has been carried out over the past, only a few agents have been found to act during the promotion stage of carcinogenesis. This study demonstrated that the administration of CKS after a NNK treatment significantly inhibited the level of NNK-induced lung tumorigenesis, as determined by the tumor multiplicity. These anti-carcinogenic actions, along with the anti-proliferation activity, suggest the potential of CKS in the chemoprevention of human cancer. Hence, this possibility should be investigated in human lung cancer prevention trials. In this study, the body weight gain was similar in the intact and NNK + CKS treated groups (data not shown). This indicates that CKS had no adverse effects on feeding or drinking. Therefore, CKS may be less toxic when used clinically. This is the first report showing that CKS effectively inhibits NNK-induced lung tumorigenesis. However, the components of CKS responsible for this inhibition require further study. Additional studies using a different animal carcinogenesis model, such as p53 transgenic mice exposed to tobacco smoke (Lubet *et al.*, 2000), might be useful for monitoring the chemopreventive ability of CKS on tobacco-related lung tumorigenesis. In conclusion, CKS has a beneficial effect on tobacco-related carcinogen NNK-induced mouse

lung tumorigenesis, might be an effective oral chemopreventive approach to lung cancer in humans.

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