Therapeutic Efficacy of Extracts from Root of Acnthopanax Sessiliflorus as Anti-cancer Drug ; *in vivo* and *in vitro* Study

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The cortex and root of Acnthopanax sessiliflorus, a herbal medicine, have been used for several diseases including cancer in Oriental countries. In the previous study, we showed that the cortex of this plant have anti-cancer activity. But its therapeutic efficacy of CORTEX ACANTHOPANAX RADICIS (CAR) is not clarified. For these reasons, we investigated immuno-potentiating and anti-cancer properties of CAR compared with CA, in terms of body and tumor weights, proliferation of thymocytes and tumor cells, and nitric oxide production from macrophages through *in vitro* and *in vivo* studies. In our results, administration of CAR reduced tumor mass and increased body weights. CAR also inhibited proliferation of tumor cells *in vivo* and *in vitro* dose-dependently. Thymocyte proliferation was accelerated by treatment with CAR and NO production was also promoted by CAR *in vivo* and *in vitro*. In conclusion, we demonstrated that CAR is useful to treat for cancer as complementary or alternative medicine to Western medication, its therapeutic efficacy is involved in direct inhibition of tumor growth and immuno-potentiating activity.

Key words : Acnthopanax sessiliflorus, herbal medicine, cancer

Introduction

In the theory of traditional medicine, The cortex and root of Acnthopanax sessiliflorus expels wind-moisture (風濕), strengthens bones and sinews (强筋骨), improves in water metabolism (利水) and attenuates turgescence (消腫). For these reason, this herbal medicine have been used to treat dull pain induced by wind-moisture (風濕痺痛), weakness of the lumbar and knee joint (腰膝軟弱), edema (水腫), and urinary abnormalites (小便不利)[1]. Based on these therapeutic efficacy, this herbal medicine have long been used for diabetes, neuralgia, palsy, gastric ulcer, learning-behavior difficulties, and cancer in oriental countries[2-5].

For recent decades, the many of biologically-active components of Acnthopanax sessiliflorus have been investigated and the compounds have been isolated. In recent years, broad investigation on several part of this plant including leaves, roots and fruits have been executed[6-9]. Ethanol extracts from this plants were known to have anti-inflammatory function[5] and polysaccharides isolated from acanthopanax senticosus were known to have phagocytosis-stimulating function[10]. In addition, it is known that Biopolymers extracted from this herbal medicine have immuno -stimulating properties[11]. In our previous studies, we elucidated that water extracts from cortex of this plant (CA, CORTEX ACANTHOPANACIS) have immuno-potentiating and anti-cancer properties[12].

In this study, we investigated immuno-potentiating and anti-cancer properties of extract from root of Acnthopanax sessiliflorus (CAR, CORTEX ACANTHOPANAX RADICIS), compared with CA, in terms of body and tumor weights, proliferation of thymocytes and tumor cells, and nitric oxide production from macrophages through *in vitro* and *in vivo* studies.

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Materials and Methods

1. Preparation of herbal medicine

The CA and CAR obtained from Acnthopanax sessiliflorus cultivated in our experimental farm was washed and cut into small pieces and then extracted with distilled water. The extract was centrifuged at 5,000 G for 30 min, then sediments were discarded. Supernatants were condensed using a Vacuum Evaporator (EYELA, Japan) and then lyophilized. Acquired extracts were alliquoted and stored at -20 ℃ before use.

2. Animals

For investigation on immuno-stimulatory effect, Eight-week-old male Balb/c mice (Damul science, Daejeon, Korea) were used. For investigation on anti-tumor effect, almost same weighed (20±1 g) ICR mice (Fine life science, Seoul, Korea) were used. Animals were housed in a temperature-controlled room (temperature 20±3 ℃, humidity 55±5 %) under a 12-hr light-dark cycle and provided with sterile food and water ad libitum. All experiments were conducted according to the institution's guidelines for care and use of laboratory animals in research.

3. Cell lines and culture conditions

The mouse peritoneal sarcoma cell line, sarcoma-180 and Mouse lymphocytic leukemia cell line, L1210 were obtained from KOREAN CELL LINE BANK (Seoul, Korea). S-180 and L1210 were maintained in RPMI 1640 (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, USA) and antibiotics (100 units/ml of penicillin, 100 ug/ul of streptomycin). Cells were incubated at 37 ℃ in a mixture of 5 % CO_2 and 95 % air.

4. Experimental design

2×10^6 of S-180 cells suspended in 0.2 ㎖ of PBS were injected into ICR mice intraperitoneally at day 1. mice were fed with 500 ㎎/kg/day of CAR or CA for 15 consecutive days and control mice were fed with vehicle for 15 days. At day 16, mice anesthetized with cervical dislocation, tumor mass was resected. Body weights were measured after resction of tumor mass. For investigation on immuno-stimulatory effect, 2×10^6 of L1210 cells suspended in 0.1 ㎖ of PBS were injected into Balb/c mice intraperitoneally. mice were fed with 500 ㎎/kg /day of CAR or CA for 7 consecutive days. At day 8, mice were anesthetized and thymus was rescted. For isolation of peritoneal macrophages, 2 ㎖ of emulsion containing 3 % thioglycollate (TG) was injected intraperitoneally at day 5.

5. Primary thymocyte and peritoneal macrophage isolation

Thymocytes were isolated from thymus according to the method of Wysocki[13]. For isolation of peritoneal macrophage, mice were anesthetized with cervical dislocation, and 10 ㎖ of cold PBS was injected intraperitoneally. Retrieved PBS containing peritoneal macrophage was centrifuged at 1,500 rpm for 5 min. Supernatants were discarded and pellets were resuspened in RPMI 1640 media. Cells were washed twice and added into 120 Ø dish, then incubated for 4 hr. After incubation, Nonadherent cells were removed by extensive washing and the remaining adherent cells were recovered by scraping.

6. Measurement of cell proliferation

Isolated thymocytes, S-180 and L1210 cells were suspened in 100 ㎕ of media and incubated into 96 well plate for 24 hr. 20 ㎖ of MTT (5 ㎎/㎖) solution was added 4 hr prior to the termination of culture. After incubation, 100 ㎕ of emulsion containing SDS (10 %) dissolved in 0.01N HCl was added in each well and incubated on light protect for 18 hr. The value of optical density (OD) was obtained by the differences in absorbance at a wavelength of 570 ㎚.

7. Measurement of NO production

Nitrite accumulation was used as an indicator of NO production. Nitrite accumulation was measured using Griess reaction[14]. Briefly, 1×10^6 cells were added into 24-well plate and incubated with 1 ㎍/㎖ LPS and 25 units/㎖ IFN-g for 24 hr. After incubation, 100 ㎕ of supernatant was mixed with an equal volume of Griess reagent [1 %(w/v) sulfanilamide and 0.2 %(w/v) naphthyl ethylenediamine in 5 % (v/v) phosphoric acid], then optical densities were measured at 570 ㎚ using Microplate-reader.

8. Statistical analysis

All statistical comparisons were made with the Student T-test. The data in this manuscript were expressed mean ± SEM. Differences with a value of $P < 0.05$ were considered significant.

Results

1. Effect of CAR on tumor size and weight *in vivo*.

At day 16, solid tumors were resected and weighed. In control group, average weight of tumor mass was 1.12±0.23 gram. Administration of CAR reduced tumor size almost 25% compared with that of control (0.85±0.06 g) (Fig. 1A). Average weight of control mice was increased by only about 78% compared with weight of day 1 (15.55±1.11 g). In CAR group, body weight was increased to about 32 % from that of control mice (22.15±26.88 g) In contrast, administration of CA not affected either tumor sizes or body weights (Fig. 1B) In normal mice, CAR did not affected body weights (data not shown).

2. Effect of CAR on proliferation of tumor cells *in vitro*.

Treatment of CAR inhibited proliferation of tumor cells such as S-180 and L1210 in vitro. proliferation of L1210 cells was inhibited by CAR dose-dependantly. The inhibition rates of L1210 were 13.80, 14.30, and 17.48 % respectively(Fig. 2A). Treatment of CAR also inhibited proliferation of S-180 cells dose-dependantly. Treatment of CAR was more effective to inhibit proliferation of S-180 than

that of L1210. The inhibition rates of S-180 were 12.41, 18.79, and 20.71 % respectively(Fig. 2B).

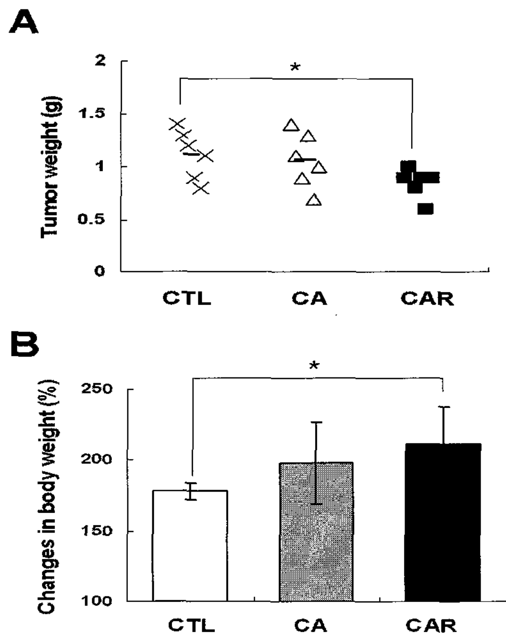

Fig. 1. Effect of CAR on tumor size and weight *in vivo*. Tumor mass and body weights were measured at the end of the experiment (day 16). Body weights were measured after resection of tumor mass. Values of tumor mass was expressed as gram (A), values of body weight are expressed as percentage of change in average weight as compared with the weight on day 1 (B). Result are presented as mean ± SEM. * P ＜ 0.05 vs. Control (n=6).

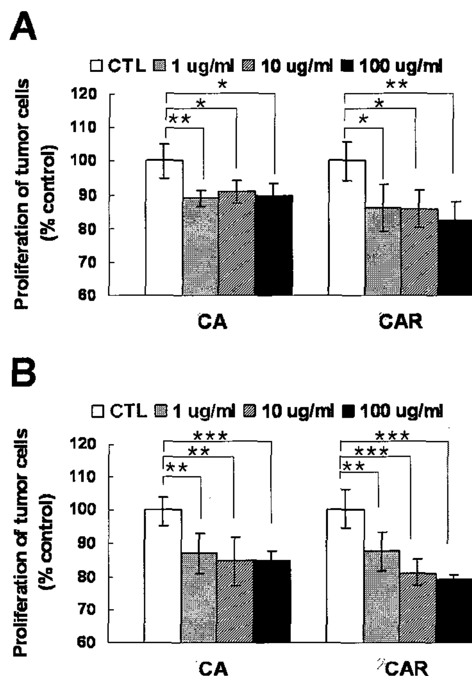

Fig. 2. Effect of CAR on proliferation of tumor cells *in vitro*. Proliferation rates of peritoneal sarcoma, S-180 (A), and lymphoblastoma, L1210 (B) were measured using MTT method. After cells were seeded and incubated for 24 h, indicated concentration of CA or CAR was added and incubated for 48 h. Values are expressed as percentage of control. Result are presented as mean ± SEM. * P ＜ 0.05 and ** P ＜ 0.01 vs. Control (n=6).

3. Effect of CAR on thymocyte proliferation in vitro.

Treatment of CAR accelerated thymocyte proliferation dose-dependantly. 1 ㎍/㎖ of CAR did not affect proliferation, but 10 ㎍/㎖, and 100 ㎍/㎖ of CAR accelerated thymocyte proliferation significantly. (about 10 % compared with control) Acceleration of thymocyte proliferation by treatment with CAR was more effective than that by CA(Fig. 3).

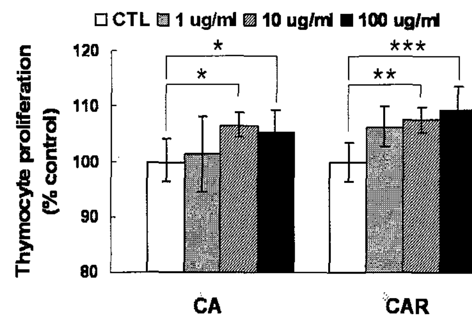

Fig. 3. Effect of CAR on thymocyte proliferation *in vitro*. Proliferation rates of primary thymocytes were measured using MTT method. After cells were seeded and incubated for 24 h, indicated concentration of CA or CAR was added and incubated for 48 h. Values are expressed as percentage of control. Result are presented as mean ± SEM. * P ＜ 0.05, ** P ＜ 0.01, and *** P ＜ 0.001 vs. Control (n=6).

4. Effect of CAR on thymocyte proliferation *in vivo*.

Thymocyte proliferation rates were estimated in normal mice and tumor bearing mice. Administration of CAR accelerated thymocyte proliferation in both normal and tumor bearing mice. (about 12% compared with control) In contrast, administration of CA did not affect thymocyte proliferation rates(Fig. 4).

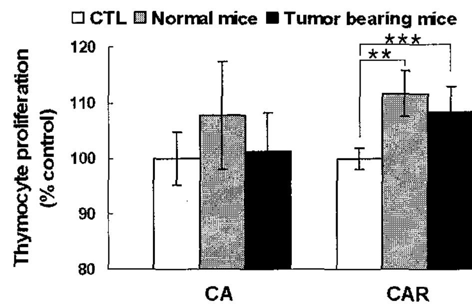

Fig. 4. Effect of CAR on thymocyte proliferation *in vivo*. Proliferation rates of primary thymocytes isolated from normal and tumor bearing mice were measured using MTT method. Mice were administrated with 500 mg/kg/day of CA or CAR for 7 consecutive days. Values are expressed as percentage of control. Result are presented as mean ± SEM. ** P ＜ 0.01 vs. Control(n=6).

5. Effect of CAR on NO production in vitro and in vivo.

1 ㎍/㎖, and 10 ㎍/㎖ of CAR promoted NO production from peritoneal macrophages in vitro. In contrast, 100 ㎍/㎖ of CAR decreased NO production level(Fig. 5A). In normal mice as well as tumor bearing mice, CAR promoted NO production significantly (about 15 % compared with control). In contrast, CA did not promote NO production in tumor bearing mice, furthermore

decreased NO production levels in normal mice(Fig. 5B).

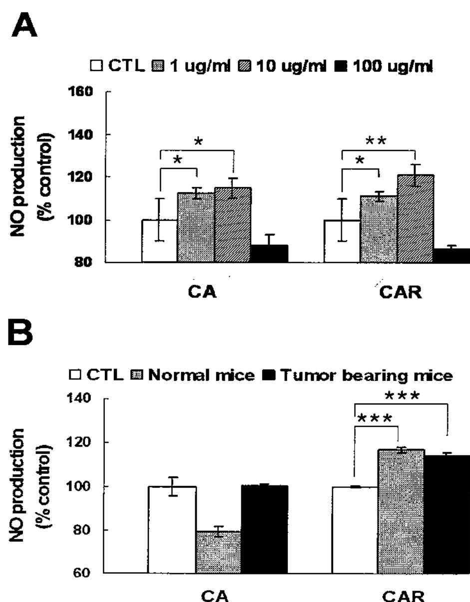

Fig. 5. Effect of CAR on NO production in vitro and *in vivo*. Isolated macrophages were incubated with 1 ug/ml LPS, 25 units/ml IFN-g and indicated concentration of CA or CAR for 24 hr in vitro. (A) In normal and tumor bearing mice, NO production from macrophages was measured. Mice were administrated with 500 mg/kg/day of CA or CAR for 7 consecutive days. (B) Values are expressed as percentage of control. Result are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. Control (n=6).

Discussion

The strategy of medication for Cancer is divided into two parts. One is direct suppression of tumor growth including Apoptosis and inhibition of proliferation, another is indirect modulation through potentiation of immune system. In this study, we investigated effect of CAR on direct suppression of tumor growth and immuno-potentiating reaction, in terms of sizes of solid tumor, proliferation of tumor cells and thymocytes, and NO production from macrophages in vivo and in vitro. Our results showed a strong possibility of CAR as anti-cancer drug. Administration of CAR reduced tumor mass in vivo, and inhibited proliferation of tumor cells in vitro. CAR promoted thymocyte proliferation in vivo, and in vitro. Finally CAR promoted NO production in vivo and in vitro study.

For several reason such as synergistic effect and safety etc., the majority of malignancies are treated with drugs in combination rather than single agents. This strategy would accelerate the acceptance of new drugs as adjunct therapies since these agents could be used at concentrations well below their maximal tolerated doses[15]. Recent interest has arisen in the use of traditional medicine as complementary or alternative medicine, possibly because of low cost and favorable safety[16]. Nonetheless, its therapeutic efficacy as well as mechanism is not clarified.

In this study, we showed that administration of CAR reduced tumor mass, conversely increased body weights. These weight gain by CAR could be attributed to improvement of general conditions and diseases. The reduction of tumor mass could be explained by the two part of cancer strategy. In our results, CAR inhibited proliferation of tumor cells and accelerated proliferation of thymocytes, which plays central roles in cell mediated immune system[17].

The stimulation of murine macrophages results in the expression of an inducible nitric oxide synthase (iNOS), which catalyzes the production of a large amount of NO from L-arginine and molecular oxygen[18]. In our results, CAR promoted NO production in vitro. CAR also promoted NO production in normal mice as well as tumor bearing mice. Our results also show that effect of CAR on immuno-potentiating reaction such as thymocyte proliferation and NO production was more effective than that of CA.

In conclusion, we demonstrates that CAR has anti-cancer activity attributed to direct inhibition of tumor growth and immune-potentiation such as acceleration of thymocyte proliferation and promotion of NO production. Further study will elucidate the exact mechanism of this potentiation and possibility as complementary medicine to typical anti-cancer drugs.

Acknowledgements

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