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Astudy on the Anticancer Activies of Lipid Soluble Ginseng Extract and Ginseng Sapongin Derivatives Against Some Cancer Cells

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인삼의 지용성 성분과 사포닌 유도체의 항암작용 연구

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Abstract

The anticancer activities of petroleum ether extract of Panax ginseng root (crude GX) and its partially purified fraction from silicic acid column chromatography (7:3 GX) were studied with Sarcoma 180 (S-180) or Walker carcinosarcoma 256 (Walker 256) in vivo and with L1210 leukemic lympocyte in vitro. Potential cytotoxic activities of the crude GX and 7:3 GX against L1210 cells were compared with those of 5-Fluorouracil (5-FU) and saponin derivatives (Panax-diol, Panax-triol, Diol saponin, Triol saponin) in vitro.

In order to observe the physiological effects of the crude GX and 7:3 GX on the animals with cancer, hemoglobin (Hb), red blood cell (R.B.C.) and white blood cell (W.B.C.) values in S-180 bearing mice or Walker 256 inoculated rats were determined after treatment with each GX in comparison with corresponding control groups, respectively. The anticancer effects of the crude GX and 7:3 GX were estimated by measuring the survival time of S-180 bearing mice after treatment with them.

The experimental results obtained are summarized as follows;

1. The one unit of cytotoxic activity against L1210 cells was equivalent to 2.54 μg and 0.88 μg of the crude GX and 7:3 GX per ml of culture medium, respectively.
2. The cytotoxic activities of Panax-diol, Panax-triol, Diol saponin and Triol saponin against L1210 cells were not detected.
3. The anticancer activities of 5-FU against L1210, S-180 and Walker 256 were very effective in vivo and vitro tests.
4. The significantly increased W.B.C. values of mice after inoculation with S-180 cells were reduced to normal range by the crude GX treatment.
5. The significantly decreased Hb values of rats after inoculation with Walker 256 were recovered to normal range by oral administration of the crude GX.
6. The survival times of mice inoculated with S-180 cells were extended about 1.5 to 2 times by the 7:3 GX treatment compared with their control group.

Introduction

Panax ginseng has been extensively used in the traditional oriental medicine as a restorative, tonic and prophylactic agent.[1] The pharmacological effect of ginseng and its extract reported during past eighteen years are various and controversial.[2-7] Very extensive pharmacological activities were reported in some purified ginsenosides with respect to central nervous system,[2] gonadotropism,[3] antistress,[4] RNA synthesis,[5] DNA, lipid and protein synthesis.[6] Both ginsenoside Rb, (protopanaxdiol group ginseng saponin) and Rg_1 (protopanaxtriol group ginseng saponin) are among the most important pharmacologically active components, but they have only part of the pharmacological properties of ginseng which have been noted.[2,7]

Recently, several reports regarding to the anticancer effects of Panax ginseng accumulated,[8-13] but these studies were incomplete as only crude extracts were used and systematic data on the anticancer active component were not included. From these studies, it was emphasized that the anticancer activities might be due to a glycoside group called ginsenoside or panax saponin in the Panax ginseng which was soluble in water.[11,12] However, we demonstrated that a highly lipid soluble component in the extract of Panax ginseng root had a considerable cytotoxic activities against some cancer cells.[13]

Therefore, this study was carried out in vitro to determine the cytotoxic activities of the lipid soluble ginseng extracts (crude GX and 7:3 GX) against L1210 cells in comparison with those of the saponin derivatives purified from Panax ginseng. Then, the crude GX and 7:3 GX which had shown very significant activities in vitro were applied in vivo test to study their effectiveness as anticancer agents or the supportive anticancer agents to 5-FU by measuring the survival time and recovery rate of the hematopoietic derangement in cancer bearing mice or rats after treatment with the ginseng extracts.

Materlals

Dried Korea ginseng roots (first grade, six year old) were purchased from a local herb shop in Seoul. Panax-diol, Panax-triol, Diol saponin and Triol saponin were a gift from the Korean Ginseng and Tobacco Research Institute. 5-Fluorouracil was purchased from drug store in Seoul. Cancer cells; murine leukemic lympocyte (L1210), murine ascitic sarcoma 180 and Walker carcinosarcoma 256 were a gift from the Research Institute for microbial Diseases, Osaka University. Experimental animals; DBA/2, C_{57}BL/6 were also a gift from the Osaka University. Swiss mice (strain A) and Albino rat (Sprague-Dawley strain) were a gift from Dr. Park (Seoul National Univ.). Each cell-line and experimental animal was maintained in our laboratory and used for the

experiment. The Fischer's medium and horse serum were purchased from the Grand Island Biological Co.; silicic acid (Bio-Sil A, 100-200 mesh) from the Bio-Rad Laboratories; millipore filter discs (GS. 0.22 μm) and accessories were from the Millipore Corp. Coulter counter (model Z_{BI}) and its accessories were goods of Coulter Electronic LTD.

Methods

Preparation and partial purification of the ginseng extracts

The Panax ginseng roots were finely pulverized using a mortar and 3 grams of the powder were subjected to extraction with petroleum ether for 12 to 15 hours by soxhlet method. The crude extract prepared from the above (crude Gx) was thoroughly dried by vacuum evaporation using a rotary evaporator under a stream of nitrogen gas and stored in a refrigerator.

The crude GX was then subjected to a silicic acid column chromatography for partial purification. After 15 g of silicic acid were activated in an oven by heating at 120°C for two hours, 50 ml of chloroform was added to it, and the slurry was poured into a glass column (2x17 cm). The prepared column was washed with 50 ml of chloroform, and then with 50 ml of petroleum ether. The crude GX (100-200 mg, dry weight) dissolved in small amount of petroleum ether was loaded to the column, and stepwise elution was processed with 50 ml of petroleum ether, 90:10 (v/v) petroleum ether-ethyl ether mixture, 80:20 (v/v) petroleum ether-ethyl ether mixture and twice with 70:30 (v/v) petroleum ether-ethyl ether mixture. From each seperated fraction, 70:30 (v/v) fraction (7:3 GX) was selected for further study, and the eluate was dried by vacuum evaporation. For the experiments, the crude GX and 7:3 GX were dissolved in small amount of absolute ethanol and diluted with water to the desired concentration.

Cell culture

L1210 cells were cultured in the Fischer's medium by Fischer & Sartorelli method.[14] Sarcoma 180 cells were maintained in Swiss mice by transplanting them every ten days after intraperitoneal injection of the cell to mice. Walker 256 cells were maintained in rat by transplanting them every ten days after hypodermic injection of the cell under chest of the Albino rat.

In vitro test; Quantification of the cytotoxicity

One unit of cytotoxic activity was arbitrarily defined as the amount of drug in one ml of culture medium which causes a two fold increase in the apparent doubling time of L1210 cells. In practice, the assay was carried out as follows. L1210 cells

were grown in media containing serial concentrations of the ginseng extracts, 5-Fu or ginseng saponin derivatives, and the cell populations of each cultured medium were counted after 24 hours of incubation by using Coulter counter. Then, the dose corresponding to the midpoint between the logarithm of control cell number at 0 hours and 24 hours of incubation was estimated from a plot of the logarithm of cell number vs. concentration of drug.

Growth curves of L1210 cells treated with different concentrations of ginseng extract or 5-FU were determined by counting the cell populations at regular intervals in the course of incubation.

Size distribution curves of L1210 cells treated with ginseng extract or 5-FU were determined in comparison with those of control cells after 0, 12 and 24 hours of incubation by using analyzer at "Manometer" or "Stop at full scale" count mode settings.

In vivo test

1) The effects of ginseng extracts or 5-FU on the survival time and hematopoietic system of S-180 bearing mice.

Fifty Swiss mice weighing from 20 to 25 g were divided into five groups (each group contained 10 mice); a normal group which received neither inoculation with S-180 cells nor other treatment, a control group inoculated with S-180 cells and three treated groups after inoculation with S-180 cell; 5-FU treated group, crude GX treated group and 5-FU & crude GX treated group. Sarcoma 180 cells were inoculated to control and each treated group of mice about $1x10^6$ cells per head by intraperitoneal injection. 5-FU (0.5 mg/head/dav) or crude GX (5.0 mg/head/day) was injected to each treated group of mice intraperitoneally for the duration of 10 days from the third day after inoculation with S-180 cells. For the 5-FU & crude GX treated group, 0.5mg of 5-FU was injected and followed by 5.0 mg of the crude GX.

Each experimental mouse was sacrificed on the fifteenth day after inoculation with S-180 cells, and then their R.B.C. and W.B.C. values were determined by using Coulter counter. Hb values were determined spectrophotometrically with Drabkin's solution at 540nm.[15]

In order to observe the effect of the crude GX on the survival time of S-180 bearing mice, another four groups of mice (control and three treated groups) were experimented in the same way as the above except sacrificing the mice on the 15th day, and the survival times of mice in each group were estimated. In addition, the effect of extended survival time was estimated again with partially purified 7:3 GX (5 mg/head/day) using another control group.

2) The effect of the crude GX on Hb values of Walker 256 bearing rats.

Seventy rats weighing about 180 g on an average were divided into seven groups

(each group contained 10 rats); a normal group which received neither inoculation with Walker 256 cells nor other treatment and six experimental groups. The experimental groups divided again into three control groups inoculated with Walker 256 cells and three treated groups with the crude GX after inoculation with Walker 256 cells. Each 10 mg of the crude GX per day was treated to rat in the treated groups by oral administration for 3,6 or 9 days, respectively.

On the 3rd, 6th and 9th day after treatment with the crude GX, each treated group and its control group was sacrificed, respectively, and Hb values of rats in each group were determined by the AOAC method.[15)]

Results and Discussion

In vitro test

The one unit of cytotoxic activity against L1210 cells was equivalent to 2.54 μg and 0.88 μg of the crude GX and 7:3 GX per ml of culture medium, respectively, so that the activity of the 7:3 GX about three times more potent than that of the crude GX (Fig. 1-A). While, the cytotoxic activities of Panax-diol. Panax-triol, Diol saponin and Triol saponin against L1210 cells were not detected in those concentractions and even with ten times higher concentrations (Fig. 1-B).

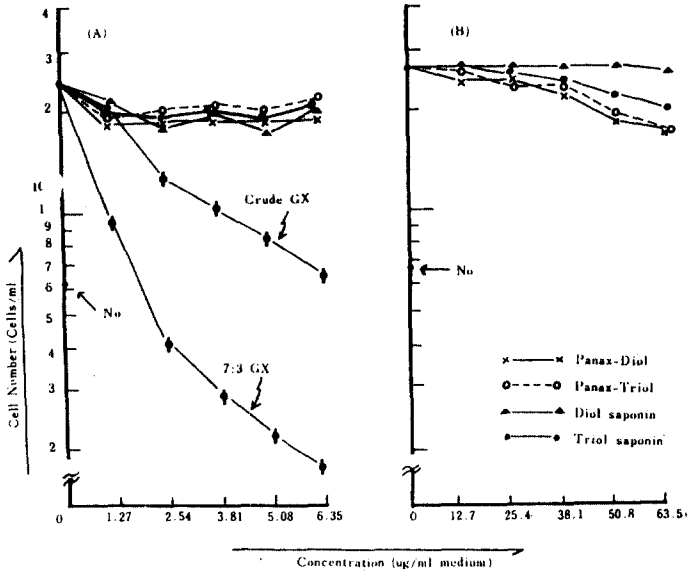

Fig 1. Dose response curves of petroleum ether extracts or saponin derivatives of Panax ginseng on the growth of L 1210 cells after 24 hours of incubation. crude GX;unpurified petroleum ether extract of ginseng root. 7:3 GX;partially purified fraction from petroleum ether extract of ginseng root by silicic acid column chromatography. saponin derivatives; Panax-Diol, Panax-Triol, Diol saponin, Triol saponin. No;initial cell number.

In previous study, we had reported about the cytotoxic activities of the crude GX against L5178Y leukemia, S-180 and HeLa cells in vitro.[13] In this present study, we have found out that the activity was also effective for another cell-line, L1210, and reconfirmed that the cytotoxic active component of the Panax ginseng was highly lipid soluble nonsaponin compound rather than saponin derivatives which had been well known to have various other pharmacological activities.[16]

Fig. 2. shows that the inhibition effects of the rude GX and 7:3 GX on the growth rate of L1210 cells in culture medium are dependent on the incubation time and concentration of the extracts. The growth inhibitions of the crude GX or 7:3 GX against L1210 cells were insignificant during the first 11 hours of incubation period but definitely significant beyond 12 hours of incubation in proportion to the dose of each extract.

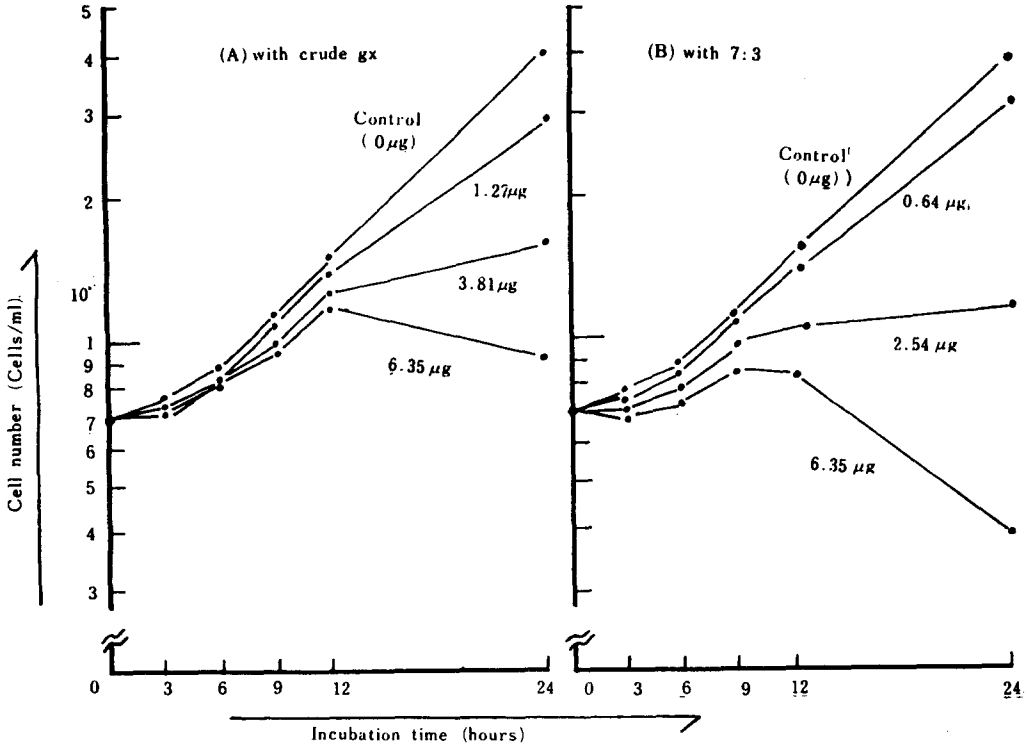

Fig 2. Growth curves of L 1210 cells in the culture medium containing various amount of the crude GX (A) and 7:3 GX (B) crude GX and 7:3 GX;see Fig 1.

Fig. 5. shows that the changes of cell size by action of the ginseng extracts in culture medium may be a factor of the decreased cell number during incubation. The overall changes of size distribution of L1210 cells treated with 6.35 μg of 7:3 GX per ml of culture medium were determined after 0,12 and 24 hours of incubation (Fig. 5-A).

Then, the peaks of each size distribution curve were determined in order to compare the size of the cells which were affected by 7:3 GX in culture medium with those of control L1210 cells (Fig. 5-B,C). The size peaks of L1210 cells incubated with the 7:3 GX were perfectly changed below 40 cubic microns (c.m.) within 24 hours of incubation, while those of the control cells were maintained at 140 c.m. during incubation. Detailed mechanism about this phenomena must be studied in view of the cell morphology.

For the comparison of the above results with already well known anticancer agent, same experiments were carried out with a synthetic pyrimidine analogue, 5-FU, which had been used frequently in the cancer chemotherapy because of its known action

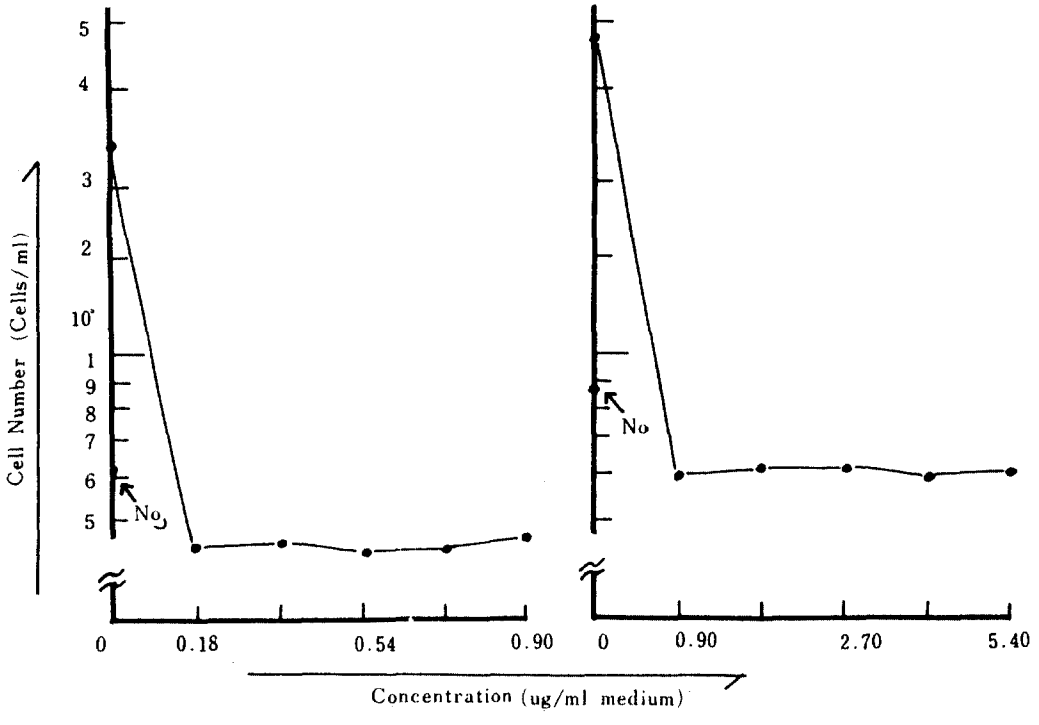

Fig 3. Dose response curves of 5-FU on the growth of L1210 cells after 24 hours of incubation
No;initial cell number.

as an inhibitor of thymidylate synthetase and RNA synthesis.[17] The dose response curves of 5-FU against L1210 cells and growth curves of the cell after treated with 5-FU showed different aspects from those of the ginseng extracts. 5-FU showed constant cytotoxic activities above its minimum dose necessary for the activity independently of its concentration (Fig. 3). Furthermore, multification of L1210 cells in a

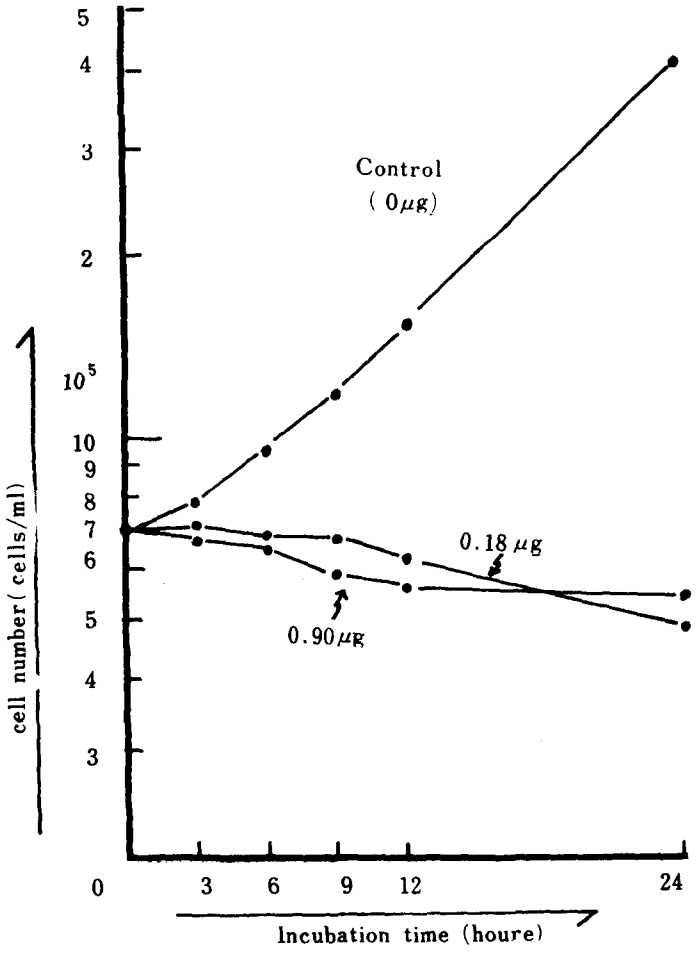

Fig 4. Growth curves of L 1210 cells in the culture medium containing 0.18 μg or 0.90 μg of 5-Fluorouracil.

culture medium was strongly inhibited by 5-FU even in the beginning of incubation (Fig. 4). As shown in Fig. 6, the shape of size distribution curves of L1210 cells affected by 5-FU was also different from that of the ginseng extract (Notice especially part B).

From the above results, it was considered that the cytotoxic mechanism of the ginseng extracts must be different from that of 5-FU.

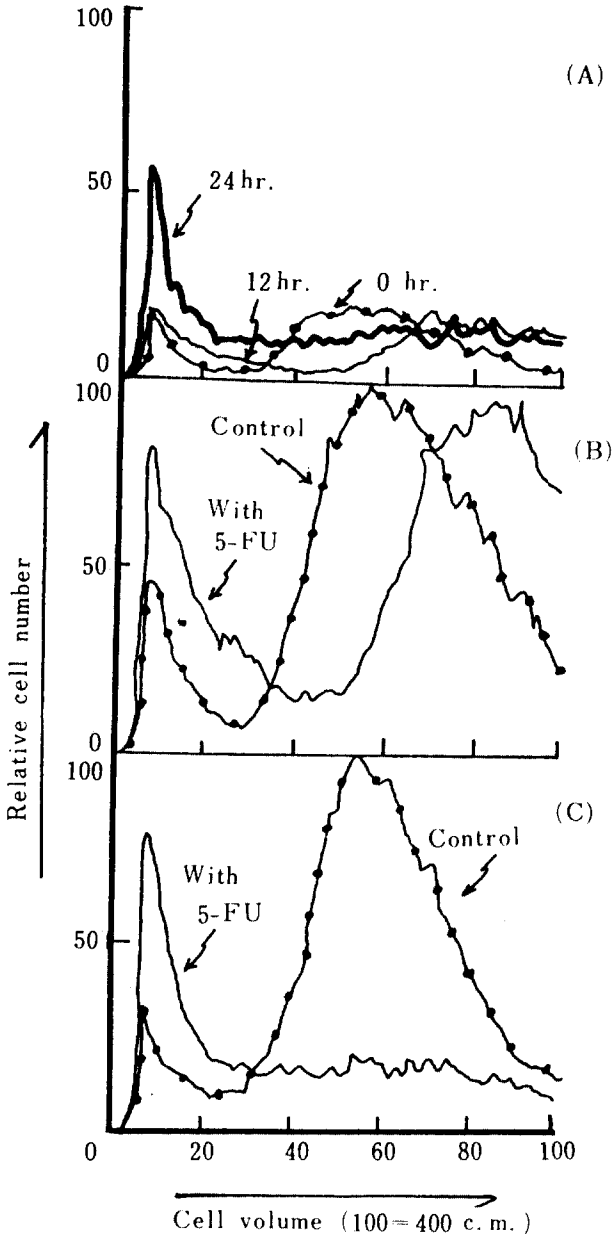

Fig 5. Size distribution curves of L 1210 cells incubated with or without 6.35 μg of 7:3 GX per ml of culture medium.

(A) Overall changes of size distribution curves of L 1210 cells incubated with 7:3 GX for 0,12 and 24 hours.

(B) Peaks of size distribution curves of L 1210 cells incubated with or without 7:3 GX for 12 hours.

(C) Peaks of size distribution curves of L 1210 cells incubated with or without 7:3 GX for 24 hours.

7:3 GX;See Fig 1.

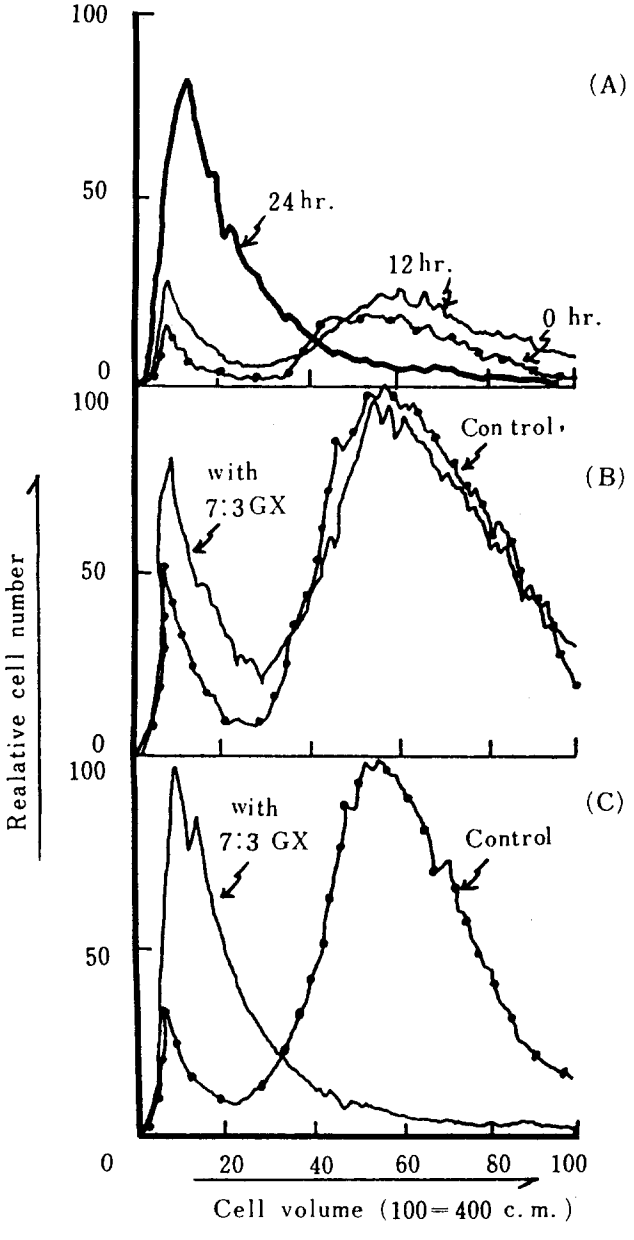

Fig 6. Size distribution curves of L 1210 cells incubated with or without 0.9 μg of 5-FU per ml of culture medium.
(A) Overall changes of size distribution curves of L 1210 cells incubated with 5-FU for 0, 12 and 24 hours.
(B) Peaks of size distribution curves of L 1210 cells incubated with or without 5-FU for 12 hours.
(C) Peaks of size distribution curves of L 1210 cells incubated with or with or without 5-FU for 24 hours.
5-FU;5-Fluorouracil

In vivo test

The R.B.C. counts ahd Hb values of S-180 bearing mice in the control group decreased somewhat compared with those of a normal group, while their W.B.C. counts increased about 3.6 times (Table 1). The crude GX was not effective in recovering R.B.C. and Hb values of S-180 bearing mice from its abnormal value but effective for W.B.C. (Table 1). 5-FU was very effective in recovering R.B.C., W.B.C. and Hb values of S-180 bearing mice from its abnormal values. However, there was no synergistic effect of 5-FU in combination with the crude GX.

Table 1. Changes of R.B.C., W.B.C. and Hb values of mice inoculated with Sarcoma-180 cells by treatment with ginseng extract or 5-Fluoro uracil for 10 day*

Group	R.B.C.($10^4/\mu l$)	W.B.C.($10^3/\mu l$)	Hb.(g %)
Normal	8.79±0.80	9.43±1.49	13.48±0.72
Control	6.69±2.03	34.10±6.60	9.05±1.67
5-FU & GX	9.27±0.83	14.84±4.55	12.47±0.81
5-FU & GX	8.30±1.85	14.47±5.61	12.45±1.59
GX	5.51±0.77	16.82±4.51	9.65±0.77

* Ginseng extract or 5-FU was injected to mice intraperitoneally for the duration of 10 days from the third day after inoculation with Sarcoma 180 cells.
Normal : Swiss mice which received neither inoculation with S-180 cells nor other treatment.
Control : Swiss mice inoculated with S-180 cells(1×10^6 cells/head).
5-FU : Treated group with 5-FU (0.5mg/head/day) after inoculation with S-180 cells.
5-FU & GX : Treated group with 5-FU (0.5mg/head/day) and crude petroleum ether extract of ginseng root (5.0mg/head/day) after inoculation with S-180 cells.
GX : Treated group with crude petroleum ether extract of ginseng root (5.0mg/head/day) after inoculation with S-180 cells.

The decreasing tendency of R.B.C.and Hb values of mice after inocularion with S-180 cells is easily understandable, because development of carcinoma can cause the hematopoietic derangement of mice.[18] The increasing tendency of W.B.C. value after inoculation with S-180 cells is also easily understandable in terms of increased production of immune bodies against invasion of S-180 cells, concurrently with the increase of lympocyte population. However, it is difficult to understand that the abnoral R.B.C., W.B.C. and Hb values of S-180 bearing mice are corrected to normal ranges by treating them with 5-FU, because 5-FU may accompany acute harmful side effect which can cause the hematopoietic derangement of mice. Therefore, these results are interpreted as the fact that the R.B.C., W.B.C. and Hb values had not been changed from the beginning due to prevention of the cancer development in mice by 5-FU rather than those values were recovered from decreased or increased values by phy-

siological action of 5-FU.

The correction of W.B.C. value from its increased level to a normal range by treating the S-180 bearing mice with the crude GX is considered to be a noticible fact, since the ginseng extract is seems to have an effect of increasing the immunity in mice.

Table 2. shows that the decrease of Hb values of rats after inoculation with Walker 256 can be prevented by treating the rats with the crude GX. In all experimental groups, significantly decreased Hb values of rats after inoculation with the cell were corrected to normal values by oral administration of the crude GX for the duration of 3,6 or 9 days. This means that the ginseng extract is expected to prevent the anemic condition caused by multification of the cancer cells in a rat.

Table 2. Changes of hemoglobin values of rats inoculated with Walker 256 cells by treatment with crude petroleum ether extract of ginseng root.*

Group		Hb(g %)
Normal		9.53±1.80
Control	1	6.79±2.33
	2	5.15±1.15
	3	6.29±1.66
GX	1	11.57±0.34
	2	12.45±1.00
	3	8.95±0.80

* Each 10 mg of crude petroleum ether extract of ginseng root per day was treated to rat by oral administration for the duration of 3, 6 and 9 days after inoculation with Walker 256 cells, respectively.

Normal : Normal rats which received neither inoculation with Walker 256 cells nor crude GX treatment.

Control 1, 2, 3 : 3-day, 6-day and 9-day group after inoculation with Walker 256 cells.

GX 1, 2, 3 : Treated group with the crude GX for 3, 6 and 9days after inoculation with Walker 256 cells.

Survival times of mice inoculated with S-180 cells were significantly increased in the 5-FU treated group or the 5-FU & crude GX treated group but very slightly increased in the crude GX treated group (Table 3-A). As the effect of extended survival time of the mice by treating them with the crude GX was not significant, another test was carried out with the partially purified 7:3 GX. About 80% of S-180 bearing mice in the 7:3 GX treated group survived more than 18 days after inoculation with the cancer cells, while none of the control group survived after 18 days (Table 3-B) On the average, the survival times of mice with the cancer were extended about 1.5 to 2 times by treating them with the 7:3 GX compared with their control group. This effect is considered to be valid even though it doesn't reach far to the effectiveness of 5-FU.

From the above results, it is concluded that the crude GX and 7:3 GX have the

Table 3. Changes of survival time of Swiss mice inoculated with sarcoma 180 cells by treatment with ginseng extracts or 5-Fluorouracil.

A) The changes by treatment with 5-FU or crude GX

Group \ Term (days)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	30	over
Control	10*		30					70					100					
5-FU	1								10				20					
5-FU & GX							10		20									
GX		10			20				60				90			100		

B) The changes by treatment with partially purified 7 : 3 GX.

Group \ Term (days)	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	30	over
Control					20		40		100									
7 : 3 GX							10				30		40	50		60	80	

* : %of dead animal.

7 : 3 GX : Treated group with partially purified fraction from petroleum ether extract of ginseng root by silicic acid column (5.0 mg/head/day) after inoculation with Walker 256 cells.

Other group : See Table 1.

potential anticancer activities against some cancer cells, but a more finely purified compound of ginseng extract must be prepared in order to establish definite anticancer effect from vivo test.

요 약

본 연구는 고려인삼 중 석유 에텔에 추출되는 crude extract와 부분 정제한 성분(7 : 3GX)의 암세포 증식억제 효과를 관찰하고자 In vitro와 In vivo에서 5-FU 및 인삼 중 saponin계 성분과 비교 실험하여 다음과 같은 결과를 얻었다.

1. In vitro test에서 crude GX와 부분 정제한 7 : 3 GX의 L1210 세포에 대한 증식 억제 활성은 각각 배양액 ml당 2.54μg 과 0.88μg 첨가시 1 unit를 나타내었다.

2. saponin계 성분(Panax-diol, Panax-triol, Diol saponin, Triol saponin)은 L1210 세포에 대한 증식 억제 효과가 없었다.

3. 5-FU는 In vitro와 In vivo 실험에서 모두 항암효과가 현저하였다.

4. Swiss mice에 Sarcoma 180 세포를 접종시 증가되었던 W.B.C. 치는 crude GX 투여에 의해 정상치에 가깝게 회복되었다.

5. Albino rat에 Walker256 세포를 접종시 감소되었던 Hb치는 crude GX 투여에 의해 정상치로 회복되었다.

6. 부분 정제한 7 : 3 GX는 Sarcoma 180 세포에 접종된 Swiss mice의 수명을 1.5-2배 연장시켰다.

이상의 결과로 보아 인삼 중 암세포 증식 억제 효과는 saponin계 성분에는 없고 지용성 성분 중에 있으며 인삼은 또한 빈혈방지 및 면역성 증진작용도 있는 것으로 사료된다.

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