Jpn. J. Cancer Res. **91**, 383–389, April 2000

Chinese Medicinal Herb, *Acanthopanax gracilistylus*, Extract Induces Cell Cycle Arrest of Human Tumor Cells *in vitro*

Bao En Shan,[1,2] Kazuya Zeki,[1] Tsutomu Sugiura,[1] Yasuhiro Yoshida[1] and Uki Yamashita[1,3]

[1]*Department of Immunology, University of Occupational and Environmental Health, School of Medicine, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555 and* [2]*Research Center, Fourth Clinical Medical College of Hebei Medical University, 5 JianKongRou, QiaoTouQu, Shijiazhung, Hebei 050011, China*

We investigated the effect of a Chinese medicinal herb, *Acanthopanax gracilistylus* (AG), extract (E) on the growth of human tumor cell lines *in vitro*. AGE markedly inhibited the proliferation of several tumor cell lines such as MT-2, Raji, HL-60, TMK-1 and HSC-2. The activity was associated with a protein of 60 kDa, which was purified by gel-filtration chromatography. Cell viability analyses indicated that the treatment with AGE inhibits cell proliferation, but does not induce cell death. The mechanism of AGE-induced inhibition of tumor cell growth involves arrest of the cell cycle at the G_0/G_1 stage without a direct cytotoxic effect. The cell cycle arrest induced by AGE was accompanied by a decrease of phosphorylated retinoblastoma (Rb) protein. Furthermore, cyclin-dependent kinases 2 and 4 (Cdk2 and Cdk4), which are involved in the phosphorylation of Rb, were also decreased. These results suggest that AGE inhibits tumor cell growth by affecting phosphorylated Rb proteins and Cdks.

Key words: Chinese medicinal herb — Anti-tumor activity — Cell cycle — Rb — Cdk

Various proteins or polysaccharides originating from higher plants have been shown to have immunopotentiating and anti-tumor activities. Chinese medicinal herbs (CMHs), mostly plants, have been traditionally used to prevent and treat many kinds of diseases in China, especially chronic diseases and tumors.[1)] Haranaka *et al*. reported that the oral administration of some CMHs to mice with transplanted tumors prolonged their survival.[2)] Wang *et al*. reported that the administration of polysaccharides from *Acanthopanax giraldii* to tumor-bearing mice prolonged survival.[3)] Kinoshita *et al*. reported that the crude extracts of *Curcumae rhizoma*, *Eucommiae cortex*, and *Cinnamomi cortex* showed anti-tumor activity against Sarcoma 180 ascites in mice, and moderately suppressed adjuvant-induced arthritis in rats.[4)] *Acanthopanax gracilistylus* (*Acanthopanax* genus) has been traditionally used as a tonic herb to treat patients with rheumatism in Chinese medicine,[1)] and has been reported to have an anti-inflammatory effect.[5)] However, other activities such as anti-tumor activity have not been investigated. In a previous paper, we reported that *A. gracilistylus* extract (AGE) had immunosuppressive activity *in vitro* and the active molecule was a 60 kDa protein.[6)] In this communication, we describe the effect of AGE on human tumor cell lines, and its effector mechanism *in vitro*.

MATERIALS AND METHODS

Preparation of CMHs CMHs were purchased from the Chinese Herbal Medicine Co. (Shijiazhung, Hebei, China). One gram of dried CMHs was steeped overnight in 100 ml of distilled water at room temperature, then boiled for 60 min. The infusion was filtered through a filter paper (Whatman type 42; Whatman International Ltd., Maidstone, UK) to remove insoluble materials and was used for experiments as a crude sample after Millipore (Milex-GP, 0.22 μm pore size ; Nippon Millipore Ltd., Tokyo) filtration.

Cell lines and cell proliferation assay Human tumor cell lines (adult T cell leukemia cell line MT-2, B cell line Raji, monocytic cell line HL-60, stomach cancer cell line TMK-1 and squamous cell carcinoma line HSC-2) were maintained by culturing in RPMI 1640 (Nissui Seiyaku Co., Yokohama) medium containing 10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY). Tumor cells (1×10^4) were cultured with or without several concentrations of CMHs in 0.2 ml of RPMI 1640 medium containing 10% FCS in wells of flat-bottomed microtiter culture plates (Falcon #3072, Becton Dickinson Co., Lincoln Park, NJ) at 37°C for 2 days in 5% CO_2 and 95% air. The cells were labeled with 0.5 μCi of tritiated thymidine ([^{3}H]TdR, specific activity 6.0 Ci/mmol, Amersham Plc, Buckinghamshire, UK) for the last 15 h and were harvested with the aid of a semiautomated cell harvester (Abe Kagaku Co., Chiba).[7)] The amount of radioactivity incorporated into DNA in the cells was measured with a liquid scintillation counter (Aloka Co., Tokyo). The results are expressed as the mean cpm of [^{3}H]TdR incorporated by cells with the SE in triplicate cultures.

Fractionation of AGE by gel-filtration Crude AGE was applied to a Sephacryl S-200 column (1.9×45 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated and eluted with phosphate-buffered saline, pH 7.4 (PBS). Ali-

[3]To whom correspondence should be addressed.
E-mail: yama-uki@med.uoeh-u.ac.jp

quots of 2 ml were collected. The protein and sugar contents in each fraction were measured in terms of absorbance (*A*) at 280 nm and by means of the phenol-sulfuric acid method,[8] respectively. The activity of each fraction was assayed in terms of the inhibition of tumor cell proliferation. The active fractions were combined, lyophilized, dialyzed and further purified on a Sephadex G-100 column (1.9×45 cm, Pharmacia Fine Chemicals). The resulting active fractions were combined, lyophilized, dialyzed and used as a purified component.

To determine the purity of AGE, crude and Sephadex G-100 fraction of AGE were separated with sodium dodecyl sulfate-polyacrylamide gel (10%, Funakoshi Yakuhin Co., Tokyo) electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue (CBB, Sigma Chemical Co., St Louis, MO).

Treatment with pronase E and $NaIO_4$ Purified AGE was incubated with 0.4 mg of pronase E (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) at 30°C in 4 ml of 0.1 *M* Tris-HCl buffer (pH 8.0) containing 50 m*M* $CaCl_2$. After 36 h incubation, 0.2 mg of pronase E was added and the incubation was continued for another 36 h. Then, the reaction mixture was heated at 100°C for 10 min to inactivate the pronase E and dialyzed against PBS.

For $NaIO_4$ treatment, purified AGE was incubated in 100 μl of 0.1 *M* $NaIO_4$ (Sigma Chemical Co.) at 25°C for 4 h. Then, 250 μl of 20% ethylene glycol (Sigma Chemical Co.) was added[9] and the sample was dialyzed against PBS.

Cell viability analysis Tumor cells (10×10^4) were cultured with or without AGE in RPMI 1640 medium containing 10% FCS in culture dishes (Falcon #3002) at 37°C for 5 days. Cells were harvested and viable cell number was counted using 0.1% trypan blue on each day.

Cell cycle analysis Tumor cells (2×10^5) were cultured with or without AGE in RPMI 1640 medium containing 10% FCS in culture dishes (Falcon #3002) at 37°C for 2 days. Cells were harvested, washed with PBS, fixed with 75% ethanol at 4°C for 2 h, then treated with 0.25 mg/ml of RNase A (Sigma Chemical Co.) at 37°C for 1 h. After having been washed, the cells were stained with 500 μg/ml propidium iodide (PI, Sigma Chemical Co.) at room temperature for 10 min. Analysis was performed on a EPICS-XL flow cytometer (Coulter Co., Healeah, FL).[10] The percentage of cells in each stage of the cell cycle was determined by using the Cellfit analysis program on the staining profile of viable cells.

Western blot analysis Western blot analysis was carried out by the method of Resnitzky *et al.* and Kaplan *et al.*[11, 12] Cell extracts were prepared, and 20 μg aliquots of the samples were subjected to SDS-PAGE (7.5% gel) and electroblotted. For detection of retinoblastoma (Rb), cyclin-dependent kinase (Cdk) 2, Cdk4 and Cdk inhibitor (p27) proteins, blots were incubated for 1 h at room temperature with 1 μg/ml anti-Rb (IF8) monoclonal antibody, anti-Cdk2 (M2), anti-Cdk4 (C-22) or anti-p27 polyclonal antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively. Thereafter, 1 h incubation was performed at room temperature with 2500× diluted horseradish peroxidase-conjugated $F(ab)'_2$ fragment of sheep anti-mouse IgG for Rb protein, or donkey anti-rabbit IgG for Cdk2, Cdk4 and p27 proteins (Amersham Pharmacia

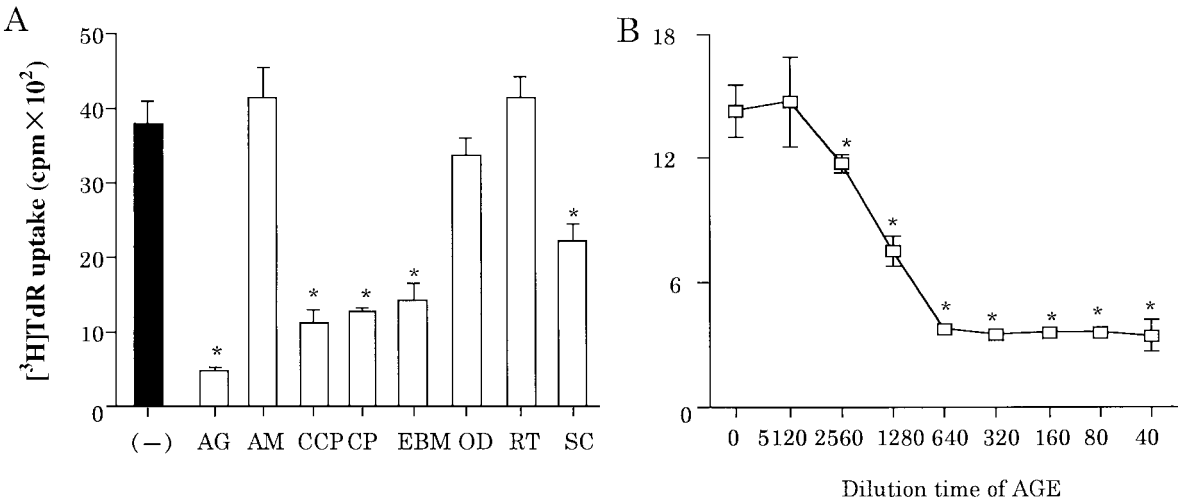

Fig. 1. Effect of eight kinds of CMHs on the growth of MT-2 cells (A). MT-2 cells (1×10^4/well) were cultured with CMHs (1:40 dilution) at 37°C for 3 days, labeled with [^{3}H]TdR for the last 15 h, and harvested, then the [^{3}H]TdR uptake by MT-2 cells was counted. Results are expressed as mean cpm of [^{3}H]TdR uptake with the SE in triplicate cultures. B shows an AGE dose-response curve for the growth inhibition. * Significantly inhibited.

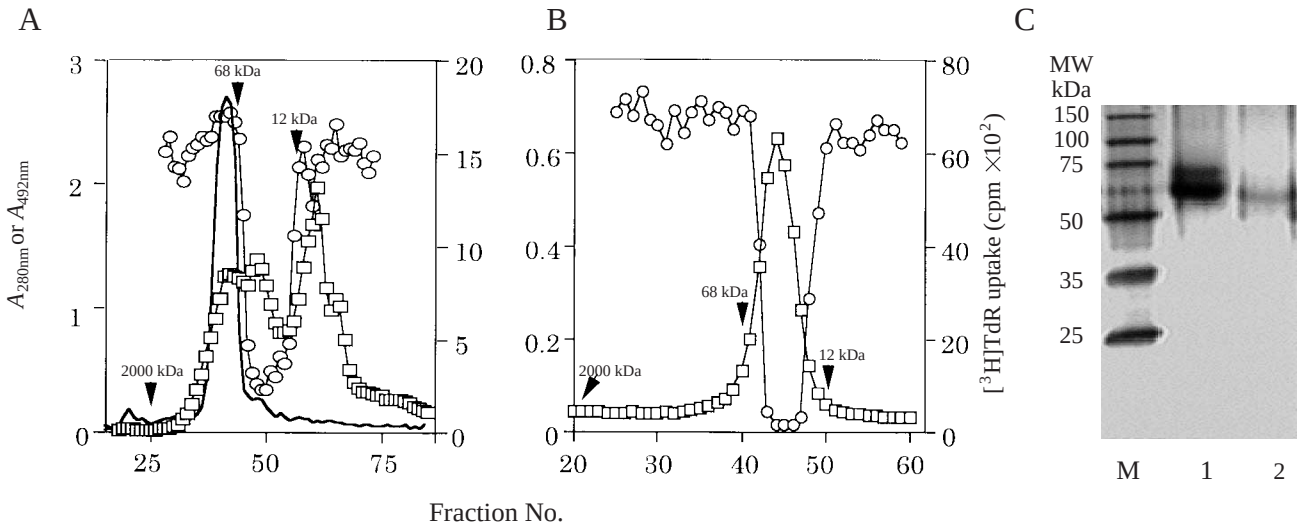

Fig. 2. Fractionation of active component of AGE. AGE was chromatographed on a Sephacryl S-200 column (A). The active fractions were combined and rechromatographed on a Sephadex G-100 column (B). The protein concentration was detected in terms of *A* at 280 nm (□). The glucose concentration was measured by the phenol-sulfuric acid method (——). The activity of each fraction was determined in terms of [^{3}H]TdR uptake (○) of MT-2 cells. The molecular size was determined by the use of marker proteins: void (blue dextran), bovine serum albumin (68 kDa) and cytochrome *c* (12 kDa). (C) SDS-PAGE of AGE. Crude and Sephadex G-100 fractions of AGE were subjected to SDS-PAGE and the bands were stained with CBB. Lane 1, crude AGE; lane 2, G-100 fraction; M, molecular weight markers.

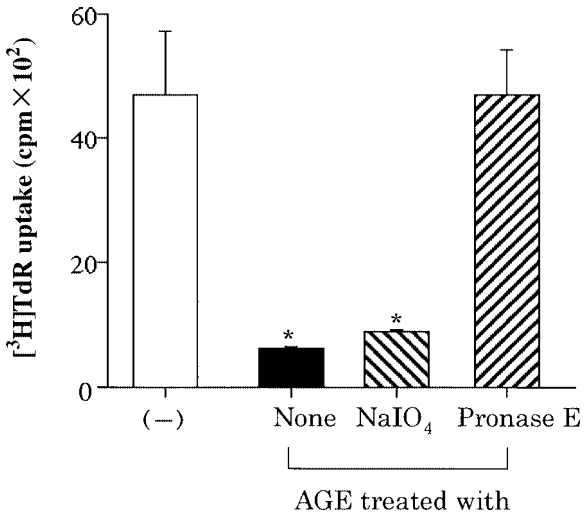

Fig. 3. Effect of pronase E and $NaIO_4$ treatment on AGE. Purified AGE was treated with pronase E (▨), $NaIO_4$ (▧) or untreated (■), and the activity was assayed in terms of the [^{3}H]TdR uptake of MT-2 cells. * Significantly suppressed.

Biotech Co., Tokyo). Finally, each protein was detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech Co.). We repeated the western blot analysis twice and obtained similar results. Representative blots of each western blot analysis are shown in "Results."

Statistics All experiments were repeated at least three times and some representative results are shown in the tables and figures. Statistical analysis was performed by using Student's *t* test. A confidence level of <0.05 was considered significant.[13)]

RESULTS

Inhibitory effect of CMHs on proliferation of MT-2 cells We first screened the effect of eight kinds of CMHs on the proliferation of MT-2 cells. As shown in Fig. 1A, the extracts of AG, *Cinnamomum cassia presl* (CCP), *Codonopsis pilosula* (CP), *Epimedium brevicornum maxim* (EBM) and *Schisandra chinensis* (SC) showed an inhibitory effect. Those of *Astragalus membranaceus* (AM), *Oldenlandia diffusa* (OD) and *Rhizoma typhonii* (RT) had no effect. Since AGE showed the strongest activity, we mainly studied AGE in this experiment. Fig. 1B shows the dose-response curve of the inhibitory effect of AGE on the proliferation of MT-2 cells. AGE dose-dependently inhibited MT-2 cell proliferation.

Active component of AGE is a protein To study the chemical nature of the active component of AGE, crude AGE was chromatographed on a Sephacryl S-200 column. The activity of each fraction was assayed in terms of the

inhibition of proliferation of MT-2 cells (Fig. 2A). The active fractions were combined, lyophilized, dialyzed and rechromatographed on a Sephadex G-100 column. The active component was eluted as a single peak whose molecular weight was about 60 kDa (Fig. 2B). The active fractions were combined, lyophilized, dialyzed and used as a purified sample. This purified fraction showed a single band at 60 kDa on SDS-PAGE (Fig. 2C). In the following experiments, we used this purified sample. This fraction was sensitive to pronase E, but not to $NaIO_4$ treatment (Fig. 3), suggesting that the effector molecule is a protein.

Inhibitory effect of AGE on proliferation of different tumor cells The inhibitory effect of AGE on proliferation of MT-2, Raji, HL-60, TMK-1 and HSC-2 cells was studied. As shown in Table I, AGE markedly inhibited the proliferation of all tumor cells studied in a dose-dependent manner. Doses required for 50% inhibition of tumor cell growth were in the range of 0.7–3.2 μg/ml protein. Fig. 4 shows the time course of the growth of tumor cells in the presence or absence of AGE. The number of tumor cells increased during culture without AGE. However, the addition of AGE decreased the number of tumor cells. There was no significant difference between the viability of tumor cells in the presence or absence of AGE. These results suggest that AGE inhibits cell proliferation without a direct cytotoxic effect.

Table I. Effect of AGE on Proliferation of Tumor Cell Lines[a)]

Cultured with AGE (μg/ml)	[^{3}H]TdR uptake				
	MT-2	Raji	HL-60	TMK-1	HSC-2
(—)	1426±126	4482±570	1903±22	5194±247	9251±1298
20	260±21*	574±67*	156±14*	362±90*	77±11*
10	314±22*	1474±167*	230±59*	366±57*	147±30*
5	348±10*	1364±57*	242±20*	768±83*	593±166*
2.5	455±20*	2360±332*	985±21*	1193±315*	1380±141*
1.25	752±70*	3836±151	1376±49*	1798±209*	2677±455*
0.65	1175±45*	4095±693	1682±49*	2587±263*	8143±369
0.31	1449±215	3485±349	1768±53*	3991±330*	8049±790
IC_{50} (μg/ml)[b)]	1.4	3.2	2.5	0.7	0.8

a) Tumor cells (1×10^4) were cultured with or without several concentrations of purified AGE at 37°C for 2 days, labeled with [^{3}H]TdR for the last 15 h, harvested and [^{3}H]TdR uptake by tumor cells was counted. Results are expressed as mean cpm of [^{3}H]TdR uptake with SE in triplicate cultures.
b) Concentrations required for 50% inhibition of cell growth.
* Significantly inhibited.

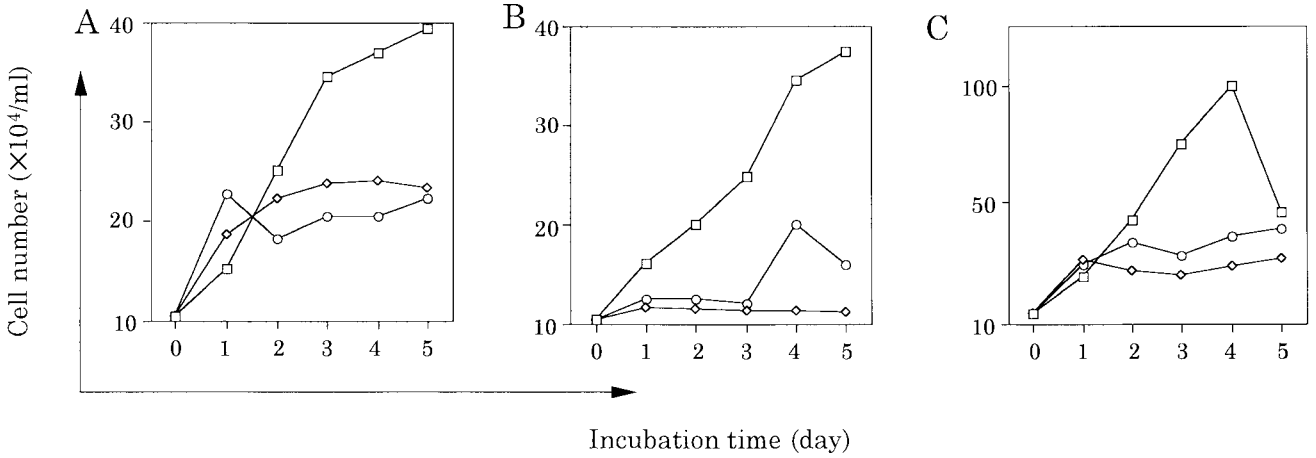

Fig. 4. Effect of AGE on growth of tumor cells. MT-2 (A), TMK-1 (B) and HSC-2 (C) (10×10^4/ml) were cultured without (□) or with 5 (○) or 10 (◇) μg/ml of AGE at 37°C for 5 days. Cells were harvested and viable cell numbers were counted using trypan blue.

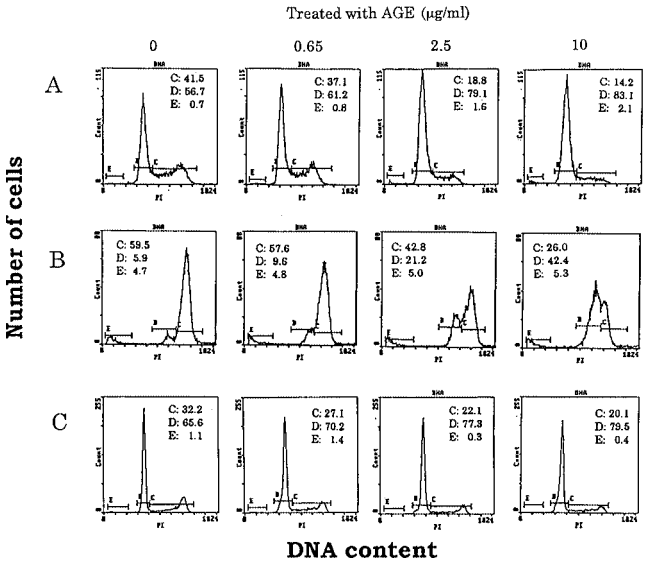

Fig. 5. Effect of AGE on cell cycle progression. MT-2 (A), TMK-1 (B) and HSC-2 (C) were cultured with or without different concentrations of AGE for 48 h, fixed and stained with PI, then the DNA content was analyzed by flow cytometry. C, S,G_2+M; D, G_0/G_1; E, dead cells. The numbers are the percentage of cells in each phase of the cell cycle.

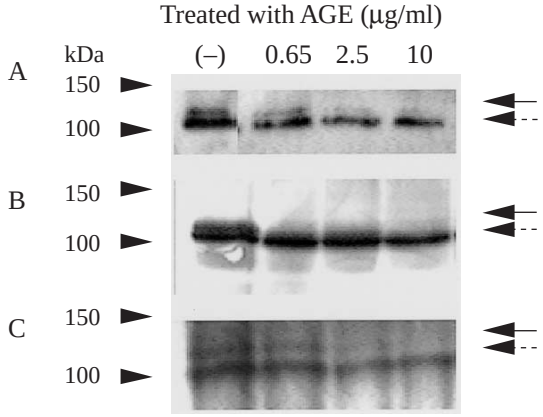

Fig. 6. Western blot analysis of expression of phosphorylated and unphosphorylated forms of Rb proteins. MT-2 (A), TMK-1 (B) and HSC-2 (C) cells were cultured without or with different concentrations of AGE for 48 h. The protein fraction was extracted, subjected to SDS-PAGE, transferred to a membrane, and blotted with anti-Rb antibody. The arrows indicate the position of the phosphorylated Rb protein, and the broken arrows indicate the position of unphosphorylated Rb protein. The numbers on the left side indicate molecular markers.

Effect of AGE on cell cycle progression To study the mechanism of AGE-induced inhibition of tumor cell proliferation, we examined the cell cycle by flow cytometry.

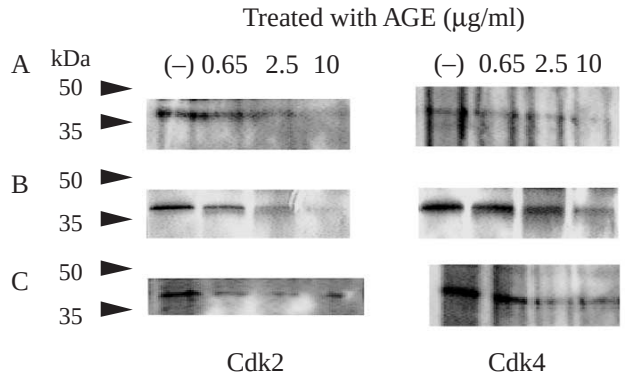

Fig. 7. Western blot analysis of expression of Cdk2 and Cdk4 proteins. MT-2 (A), TMK-1 (B) and HSC-2 (C) cells were cultured without or with different concentrations of AGE for 48 h. The protein fraction was extracted, electrophoresed, transferred to a membrane and blotted with anti-Cdk antibody. The numbers on the left side are molecular markers.

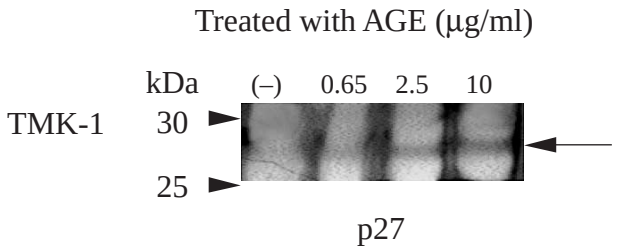

Fig. 8. Western blot analysis of expression of Cdk inhibitor. TMK-1 cells were cultured without or with AGE and the same procedure as described in the legend to Fig. 7 was performed, using anti-p27 antibody.

Tumor cells were cultured with or without AGE for 48 h, washed, and stained with PI, then the cell cycle was analyzed. As shown in Fig. 5A, in untreated MT-2 cells, 56.7% were at G_0/G_1 phase and 41.5% were at S or G_2+M phase of the cell cycle. After AGE treatment, G_0/G_1-phase cells increased to 83.1% and S or G_2+M-phase of cells decreased to 14.2%. The fraction of apoptotic and necrotic cells was not increased by AGE. TMK-1 (Fig. 5B) and HSC-2 cells (Fig. 5C) gave similar results. Thus, it appears that AGE inhibits cell cycle progression by causing arrest at the G_1 phase, but does not induce cell death.

Effect of AGE on Rb and Cdk proteins Progression of the cell cycle is controlled by several Cdks which regulate the activity of regulatory proteins of the Rb family.[14, 15] Because the growth arrest is generally associated with a reduction in phosphorylated Rb protein, we examined the effect of AGE on Rb protein. As shown in Fig. 6A, the treatment of MT-2 cells with AGE induced a reduction of

the phosphorylated form of Rb (p116) protein. However, the unphosphorylated form (p110) protein was not reduced. Reduction of phosphorylated Rb protein by AGE was also observed in TMK-1 (Fig. 6B) and HSC-2 cells (Fig. 6C).

Cdk2 and Cdk4 phosphorylate Rb protein late in the G_1 phase. We next examined whether the reduction of phosphorylated Rb protein was related to the reduction of Cdk proteins. As shown in Fig. 7A, the treatment of MT-2 cells with AGE induced the reduction of Cdk2 and Cdk4 proteins. The reduction of Cdk2 and Cdk4 proteins by AGE treatment was also observed in TMK-1 (Fig. 7B) and HSC-2 cells (Fig. 7C). The expression of Cdk inhibitor (p27) was enhanced by AGE (Fig. 8). These results suggest that AGE inhibits cell cycle progression by affecting Rb and Cdk proteins.

DISCUSSION

The effects of CMHs on the growth of tumor cells have been examined in animal models by several investigators.[1–3] *Acanthopanax senticosus* is a CMH which has been used to treat patients with rheumatism. Recently, AG also has been reported to have an anti-inflammatory effect,[5] though its other activities such as anti-tumor activity and its chemical nature have not been fully established. In this communication, we report a novel activity of AG that induces growth arrest in several human tumor cell lines *in vitro*.

We first screened the anti-tumor activity of eight kinds of CMHs. Five of them showed anti-proliferative activity towards tumor cells. Since AGE showed the strongest activity, we mainly studied the active component and effector mechanism using AGE. The active component of AGE was purified by sequential gel-filtration on Sephacryl S-200 and Sephadex G-100 columns. It is a 60 kDa protein which is sensitive to pronase E treatment, but not to $NaIO_4$ treatment. AGE exhibited activity against not only leukemic cell lines, such as MT-2, Raji, HL-60, but also epithelial cell lines, such as stomach cancer cell line TMK-1 and squamous cell carcinoma line HSC-2.

To study the mechanism of the anti-tumor activity of AGE, the effect of AGE on cell viability and the cell cycle was investigated. AGE inhibited the proliferation of tumor cells without affecting their viability. Analysis of the DNA content of cells using PI demonstrated that AGE blocked the transition of cells from G_1 to S-phase of the cell cycle within 48 h of the treatment. This suggests that AGE induces the arrest of cell cycle at the G_0/G_1 stage without inducing apoptosis or necrosis of tumor cells.

The mechanisms of cell cycle progression and arrest have been extensively studied, and the involvement of Rb proteins such as p110 and p116[16] and also of several Cdks in the G_1 phase has been reported.[15] Rb proteins play important roles in the control of progression through the G_1 stage of the cell cycle.[14, 15, 17] In early G_1, unphosphorylated Rb proteins are present as a complex with the transcription factor E2F, thereby inactivating E2F. The phosphorylation of Rb proteins in the mid-to-late G_1 phase owing to the action of Cdk:cyclin complexes results in dissociation of the Rb:E2F complex and allows E2F to activate transcription of several genes, such as *cyclin A*, *thymidine kinase* and c-*myc*, which are required for the progression through late G_1 and into S phase of the cell cycle. Our results demonstrate that treatment with AGE results in a decrease of phosphorylated Rb proteins in several human tumor cell lines, while no change was observed in unphosphorylated Rb proteins. In addition, we found that AGE treatment causes a reduction of Cdk2 and Cdk4 proteins and an enhancement of Cdk inhibitor (p27). Therefore, AGE-induced G_1 arrest seems to be mediated by the effect on Rb and Cdk proteins.

Another important finding in this study is that AGE has inhibiting activity on not only leukemic cells, but also tumor cells from epithelium of the gastrointestinal tract. This means that AGE might be applicable by oral administration.

Several proteins or polysaccharides originating from higher plants were reported to have activities as biological response modifiers.[3, 18–26] *A. senticosus*, belonging to the same genus as AG, as well as its polysaccharide fraction, enhanced the phagocytic activity of macrophages and inhibited the growth of transplanted tumors in mice.[2, 21, 22] In a previous paper we studied the effect of AGE on immune responses and found that AGE had immunosuppressive activity. However, the immunosuppressive activity of AGE showed cellular specificity. AGE inhibited T cell and B cell functions, while it stimulated macrophages to produce cytokines and inhibited tumor cell growth.[6]

Although several questions, such as the precise chemical nature of AGE, its binding sites on tumor cells and its signal transduction mechanism in the cells, still remain unresolved in this study, the anti-tumor activity and immunopotentiating activity provide a rational basis for the clinical efficacy of this medicinal herb. Further purification of active components and examination of the effects *in vivo* are in progress.

(Received October 30, 1999/Revised February 2, 2000/Accepted February 7, 2000)

REFERENCES

1) Li, X. Y. Immunomodulating Chinese herbal medicines. *Mem. Inst. Oswaldo Cruz*, **86**, 159–164 (1991).
2) Haranaka, K., Satomi, N., Sakurai, A., Haranaka, R., Okada, N. and Kobayasi, M. Antitumor activity of traditional Chinese medicines and crude drugs. *Cancer Immunol. Immunother.*, **20**, 1–5 (1985).
3) Wang, J. Z., Tsumura, H., Shimura, K. and Ito, H. Antitumor activity of polysaccharide from a Chinese medicinal herb, *Acanthopanax giraldii Harms*. *Cancer Lett.*, **65**, 79–84 (1992).
4) Kinoshita, G., Nakamura, F. and Maruyama, T. Immunological studies on polysaccharide fractions from crude drugs. *Jpn. J. Herb. Med.*, **40**, 325–332 (1986) (in Japanese).
5) Tang, X., Ma, Y. and Li, P. Separation and identification of the anti-inflammatory diterpene from the root cortices of *Acanthopanax gracilistylus*. *J. Chin. Herb. Med.*, **20**, 231–253 (1995) (in Chinese).
6) Shan, B. E., Yoshida, Y., Sugiura, T. and Yamashita, U. Suppressive effect of Chinese medicinal herb, *Acanthopanax gracilistylus*, extract on human lymphocytes *in vitro*. *Clin. Exp. Immunol.*, **119**, 41–48 (1999).
7) Harrison, M. R., Thurmond, G. and Thomas, G. M. A simple and versatile harvesting device for processing radioactive label incorporated into and/or released from cells in microculture. *J. Immunol. Methods*, **4**, 11–20 (1974).
8) Dubis, M., Gillis, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356 (1962).
9) Oka, S., Shigeta, S., Ono, K. and Jyo, T. An epitope residing in carbohydrate chains of sea squirt antigen termed Girep. *J. Allergy Clin. Immunol.*, **80**, 57–63 (1987).
10) Gallardo, S., Cardaba, B., Pozo, V., Amdres, B., Cortegano, I., Jurado, A., Tramon, P. P. and Lahoz, C. Study of apoptosis in human lymphocytes by toxic substances implicated in toxic oil syndrome. *Toxicology*, **118**, 71–82 (1997).
11) Resnitzky, D., Tiefenbrun, N., Beriss, H. and Kimchi, A. Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth sensitive hematopoietic cells. *Proc. Natl. Acad. Sci. USA*, **89**, 402–406 (1992).
12) Kaplan, M. H., Daniel, C., Schindler, U. and Grusby, M. J. Stat proteins control lymphocyte proliferation by regulating $p27^{kip1}$ expression. *Mol. Cell. Biol.*, **18**, 1996–2003 (1998).
13) Zar, J. H. "Biostatistical Analysis" (1974). Prentice Hall Inc., Englewood Cliffs, NJ.
14) Dyso, N. pRb, p107 and the regulation of the E2F factor. *J. Cell Sci.*, **18**, 81–87 (1994).
15) Sher, C. J. and Roberts, J. M. Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163 (1995).
16) Knudsen, E. S., Pazzagli, C., Born, T. L., Knudsen, K. E., Arden, K. C., Henry, R. R. and Feramisco, J. Elevated cyclins and cyclin-dependent kinase activity in the rhabdomyosarcoma cell line RD. *Cancer Res.*, **58**, 2042–2049 (1998).
17) Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704 (1993).
18) Lan, Z. F., Cheng, G. Q., Wang, F. L. and Xi, S. F. Effects of *Rodix hedysari* polysaccharide on immunological function and transplanted tumor in mice. *Chin. Pharmacol. Acta*, **8**, 257–277 (1987).
19) Franz, G. Polysaccharides in pharmacy: current applications and future concepts. *Planta Med.*, **55**, 493–497 (1989).
20) Shimura, K. and Ito, H. Screening of host-mediated antitumor polysaccharides by crossed immunoelectrophoresis using fresh human serum. *Jpn. J. Pharmacol.*, **33**, 403–408 (1983).
21) Fang, J. N., Proksch, A. and Wagner, H. Immunologically active polysaccharides of *Acanthopanax senticosus*. *Phytochemistry*, **24**, 2619–2622 (1985).
22) Xie, S. S. Immunoregulatory effect of polysaccharide of *Acanthopanax senticosus* (PAS). Immunological mechanism of PAS against cancer. *J. Chin. Med.*, **11**, 338–340 (1989) (in Chinese).
23) Shen, M. L., Zhai, S. K., Chen, H. L., Luo, Y. D., Tu, G. R. and Ou, D. W. Immunopharmacological effect of polysaccharides from *Acanthopanax senticosus* on experimental animals. *Int. J. Immunopharmacol.*, **13**, 549–554 (1992).
24) Chu, D. T., Wong, W. L. and Mavligit, G. M. Immunotherapy with Chinese medicinal herbs. I. Immune restoration of graft-versus-host reaction in cancer patients by fractionated *Astragalus membranaceus in vitro*. *J. Clin. Lab. Immunol.*, **25**, 119–123 (1988).
25) Yoshida, Y., Wang, M. Q., Liu, J. N., Shan, B. E. and Yamashita, U. Immunomodulating activity of Chinese medicinal herbs and *Oldenlandia diffusa* in particular. *Int. J. Immunopharmacol.*, **19**, 359–370 (1997).
26) Shan, B. E., Yoshida, Y., Sugiura, T. and Yamashita, U. Stimulating activity of Chinese medicinal herbs on human lymphocytes *in vitro*. *Int. J. Immunopharmacol.*, **21**, 149–159 (1999).