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

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We investigated the effect of a Chinese medicinal herb, *Acanthopanax gracilistylus* (AG), extract (E) on the growth of human tumor cell lines in *vitro*. AG 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 G1/G0 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 Cdns.

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 *Curcuma rhizoma*, *Eucamomiae 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,† 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. (Shijiazhuang, 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 T MK-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 (1×10⁵) 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% CO₂ and 95% air. The cells were labeled with 0.5 μCi of tritiated thymidine ([3H]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). 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 [3H]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-
quotients 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, 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 NaIO₄** 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 mM CaCl₂. 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 NaIO₄ treatment, purified AGE was incubated in 100 µl of 0.1 M NaIO₄ (Sigma Chemical Co.) at 25°C for 4 h. Then, 250 µl of 20% ethylene glycol (Sigma Chemical Co.) was added and the sample was dialyzed against PBS.

**Cell viability analysis** Tumor cells (10"×10⁴) 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 (2×10⁵) 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).

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. 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 horse-radish 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 Biotech, Buckinghamshire, England). Results are expressed as mean cpm of [³H]TdR uptake in triplicate cultures. B shows an AGE dose-response curve for the growth inhibition. * Significantly inhibited.

![Fig. 1](image-url)

Fig. 1. Effect of eight kinds of CMHs on the growth of MT-2 cells (A). MT-2 cells (1×10⁴/well) were cultured with CMHs (1:40 dilution) at 37°C for 3 days, labeled with [³H]TdR for the last 15 h, and harvested, then the [³H]TdR uptake by MT-2 cells was counted. Results are expressed as mean cpm of [³H]TdR uptake with the SE in triplicate cultures. B shows an AGE dose-response curve for the growth inhibition. * Significantly inhibited.
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 Sepharlcy S-200 column. The activity of each fraction was assayed in terms of the

| Fraction No. | AβC | AβA | AβA280nm | AβA492nm |
|--------------|-----|-----|---------|---------|
| 1            |     |     | 2000 kDa| 12 kDa  |
| 2            |     |     | 68 kDa  |         |
| M            |     |     |         |         |

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 measured by the phenol-sulfuric acid method (——). The activity of each fraction was determined in terms of [3H]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 NaIO4 treatment on AGE. Purified AGE was treated with pronase E ( ), NaIO4 ( ) or untreated ( ), and the activity was assayed in terms of the [3H]TdR uptake of MT-2 cells. * Significantly suppressed.
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 NaIO₄ 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

| Cultured with AGEl/µg/ml | [3H]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*      | 587±263*      | 8143±369      |
| 0.31                     | 1449±215      | 3485±349      | 1768±53*      | 3991±330*     | 8049±790      |

IC₅₀(µg/ml) 1.4 3.2 2.5 0.7 0.8

a) Tumor cells (1×10⁴) were cultured with or without several concentrations of purified AGE at 37°C for 2 days, labeled with [3H]Tdr for the last 15 h, harvested and [3H]Tdr uptake by tumor cells was counted. Results are expressed as mean cpm of [3H]Tdr uptake with SE in triplicate cultures.

b) Concentrations required for 50% inhibition of cell growth.

* Significantly inhibited.
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. Tumor cells were cultured with or without AGE for 48 h, fixed and stained with PI, then the DNA content was analyzed by flow cytometry. C, S, G2+M; D, G0/G1; E, dead cells. The numbers are the percentage of cells in each phase of the cell cycle.

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 phosphorylated Rb protein. The numbers on the left side indicate molecular markers.

Effect of AGE on Cdk2 and Cdk4 proteins

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.

Effect of AGE on Cdk inhibitor

TMK-1 cells were cultured with or without AGE and the same procedure as described in the legend to Fig. 7 was performed, using anti-p27 antibody.
The mechanisms of cell cycle progression and arrest have been extensively studied, and the involvement of Rb proteins such as p110 and p116, and also of several Cdks in the G1 phase has been reported. Rb proteins play important roles in the control of progression through the G1 stage of the cell cycle. In early G1, 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 G1 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 G1 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 G1 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. 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. 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.

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)
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