

Effects of astragali radix on the growth of different cancer cell lines

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Abstract

AIM: To investigate the inhibitory effect of a Chinese herb medicine Astragali radix (AR) on growth of different cancer cell lines.

METHODS: To observe the in vitro effects of AR on tumor cell proliferation by trypan blue exclusion, MTS method and tritium thymidine incorporation assay. Apoptosis was detected by DNA ladder method.

RESULTS: The inhibition rates of AR on the cell respiration of AGS, KATOIII, HT29, MDA231, MEL7 and MEL14 were 68.25 %, 62.36 %, 22.8 %, 27.69 %, 2.85 % and 5.14 % respectively at the concentration of 100 ug/ml; it inhibited AGS DNA synthesis by 87.33 % at the concentration of 50 ug/ml. The inhibitory effect on AGS was time- and dose-dependent. AR did not induce apoptosis in AGS cells.

CONCLUSION: AR specifically inhibits gastric cancer cells growth in vitro and the mechanism is mainly cytostatic but not cytotoxic or inducing apoptosis.

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INTRODUCTION

Astragali radix (AR) is the dried root of *Astragalus membranaceus* Bge. Var. *mongholicus* and is used as a tonic in the traditional Chinese medicine. It has been used extensively as an adjuvant^[1,2] in cancer treatment and as a phytochemical immune modulator. Kurashige *et al.* reported that AR lowered the incidence of urinary bladder carcinoma in N-butyl-N'-butanolinitrosoamine treated mice by activating the cytotoxicity of lymphocytes and increasing the production of IL-2 and IFN- γ ^[3]. Lau's study showed that it also restored the chemiluminescent oxidative burst activity of murine splenic macrophage suppressed by renal cell carcinoma^[4]. Wang's research suggested that an extract of AR had the synergetic effect with IL-2 in activating LAK cells, resulting in reducing the dosage of IL-2 and the associated toxicity^[5]. In addition, AR also could promote the proliferation of B cell and the production of immunoglobulin^[6] and has a bidirectional modulating effect on T cells. It was reported that it could reduce

the suppressive activity of Ts in post-burn mice^[7] and also increase Th cell activity in immunodepressed mice^[8].

However, there was no report on whether AR affects tumor cells growth directly. In this paper, we studied the effect of an aqueous extract of AR on the growth of different cancer cell lines.

MATERIALS AND METHODS

Cell lines and culture conditions

Human gastric cancer cell lines AGS and KATO-III were purchased from the American Type Culture Collection. AGS is a cell line of moderately-poorly differentiated adenocarcinoma and KATO-III is a cell line of signet ring carcinoma. HT29 is a cell line of colon cancer and MDA231 is a breast cancer cell line, both of them were kindly provided by Dr. Bill Murphy. Mel7 and Mel14 are melanoma cell lines, which were gifted by Dr. D. Schadendorf. All the cells were cultured in RPMI1640 medium supplemented with 10 % FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and Glutamine (GIBCO BRL, Life Technologies, Grand Island, NY, USA) in a humidified atmosphere of 95 % air with 5 % CO₂ at 37 °C as a monolayer.

Preparation of herbal extract

AR was purchased from Da Xing Chinese herbal medicines store in DC, USA. The aqueous extract was prepared by the Natural Product Branch of National Cancer Institute. 8 grams of aqueous extract was dissolved in 100 ml distilled water and centrifuged at 4 000 rpm for 20 min. The supernatant was passed through a 0.22 μ m filter (Corning, Costar, NY, USA) and reached a final concentration of 80 mg/ml. The solution was aliquoted and stored at -20 °C for future use.

AR treatment

The herbal treatment was modified from the anti-cancer drug screening program of natural product branch in NCI^[9]. Cells were harvested by trypsinization when they were confluent. 1×10^4 and 3×10^5 cells were seeded in each of the 96-well and 6-well plates respectively and cultured in RPMI 1640 medium supplemented with 5 % FBS for 24 hours. Then different concentrations of AR were added in each well to attain a series of different concentrations (1, 12.5, 25, 50 and 100 μ g/ml) and incubated for 48 hours for further measurements.

MTS assay

Cell growth was measured by MTS assay. The Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit was purchased from Promega, WI, USA. Briefly, MTS and PMS were mixed at the ratio of 20:1 immediately before being added to the samples. 20 μ l of MTS/PMS solution was added to each of the 96-well plate and incubated at 37 °C in a humidified 5 % CO₂ atmosphere for 3 hours. The absorbance was read at 490 nm using Bio-Tek's Power Wave x reader-assay system (BIO-TEK Instruments INC, VT, USA). Each sample was triplicated.

³H-thymidine incorporation assay

Cell DNA synthesis was measured by ³H-thymidine incorporation assay. At the end of herbal treatment, 1 μ Ci of ³H-thymidine was added in each well of a 96-well plate. Then the plate was incubated at 37 °C in a humidified 5 % CO₂

atmosphere for 4 hours and stored at -70°C overnight. On the second day, the frozen plate was thawed at 37°C and the DNA was transferred to the filtermat using multi-channel cell collector. The filtermat was washed with distilled water thrice and 95 % alcohol once. 4.5 ml Betaplate Scint was added to the filtermat and read the filtermat with liquid scintillation and luminescence counter (Perkin Elmer Wallac Inc. MD, USA). Each sample was quadruplicated.

Cell viability assay

Cells were cultured and treated in 6-well plate. At the end of the treatment, the cells were collected by trypsinization and counted with 1 % Trypan-blue. Each sample was triplicated.

DNA ladder analysis

DNA ladder was measured as described previously^[10] with some modification. Briefly, 1×10^6 cells in 0.5 ml were lysed in 1 ml DNA lysis buffer containing 1 M Tris-HCl, 0.2 M EDTA, 4 M NaCl, 20 % SDS and 400 $\mu\text{g}/\text{ml}$ proteinase K) and incubated at 37°C overnight. DNA was extracted in Phase LockGel eppendorf (Eppendorf Scientific Inc. NY, USA) with an equal volume of phenol/choloroform/isoamyl alcohol (25:24:1) and precipitated with 2 volumes of ice-cold 100 % ethanol and 1/10 volume of 3 M sodium acetate at -70°C for 1 hr. DNA was collected, washed with ice-cold 70 % ethanol once and dried in air. Then DNA was dissolved in TE buffer containing 10 $\mu\text{g}/\text{ml}$ RNase I and incubated at 37°C for 1 hr. Equal amounts of DNA (10 $\mu\text{g}/\text{well}$) were electrophoresed in 1.8 % agarose gels impregnated with ethidium bromide (0.1 $\mu\text{g}/\text{ml}$) for 2 hr at 70 V. DNA fragments were visualized by UV transillumination.

Statistical analysis

The inhibition rate was calculated by the following formula: inhibition rate (%) = $(\text{OD}/\text{CPM}_{\text{control}} - \text{OD}/\text{CPM}_{\text{test}}) / \text{OD}/\text{CPM}_{\text{control}} \times 100\%$. Student's *t*-test was used to compare the results. All *P* values were described by two-tailed analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Growth inhibiting effect of AR on different cancer cell lines

Figure 1 illustrated the inhibiting effects of AR on the proliferation of 6 tumor cell lines, including AGS, KATOIII, HT29, MDA231, MEL7 and MEL14, measured by MTS method. It was found that after 48 hours incubation, 100 $\mu\text{g}/\text{ml}$ of AR had greater inhibiting effect on gastric tumor cell lines of AGS and KATOIII than the other cancer cell lines. It inhibited the growth of AGS and KATOIII by 68.25 % and 62.36 % respectively, compared to only 22.8 %, 27.69 %, 2.85 % and 5.14 % reduction in cell growth of HT29, MDA231, MEL7 and MEL14. It appeared to be selectively inhibiting the growth of gastric cancer cell line.

Effect of AR on AGS viability

Whether the inhibitory effect of AR on AGS growth was cytotoxic was determined by the trypan blue measurement. Figure 2 showed the viable cell numbers of AGS treated with different concentrations of AR. In the range from 1 to 50 $\mu\text{g}/\text{ml}$, AR had a dose-dependent inhibiting effect on AGS growth and almost completely inhibited the AGS growth at the concentration of 50 $\mu\text{g}/\text{ml}$, of which the cell number of the test group was very close to the cell number seeded at the beginning of the test and few cells were stained blue. However, at the concentration of 100 $\mu\text{g}/\text{ml}$, AR showed cytotoxicity on AGS, of which the cell number was lower than the initial cell number.

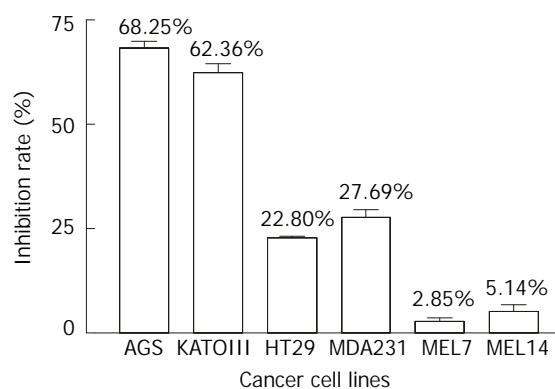


Figure 1 Effect of AR on the growth of 6 cancer cell lines. Cells were treated with AR at the concentration of 100 $\mu\text{g}/\text{ml}$ for 48 hrs and cell growth was measured with MTS method.

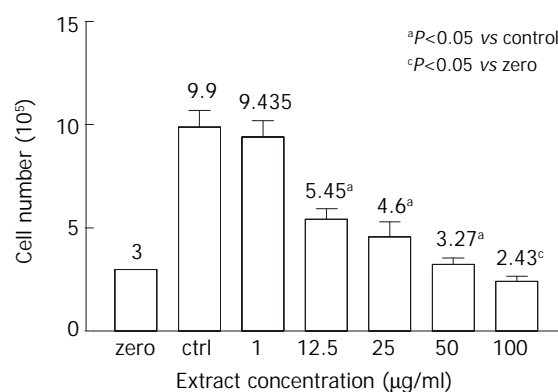


Figure 2 Effect of AR on AGS viability. AGS cells were treated with different concentrations of AR for 48 hrs and cell viability was measured with trypan blue. At or below the concentration of 50 $\mu\text{g}/\text{ml}$, AR was not cytotoxic to AGS cells. But at the concentration of 100 $\mu\text{g}/\text{ml}$, it showed cytotoxicity on AGS.

Dose-dependent and time-dependent effects of AR on AGS growth

The dose-dependent and time-dependent effects of AR on AGS growth were observed by MTS assay. AGS cells were treated with different concentrations of AR for 48 hours. Figure 3 showed that the inhibitory effect of AR on AGS was proportional to the extract concentrations. In the time-course experiment, AGS cells were treated with 50 $\mu\text{g}/\text{ml}$ of AR and incubated for 6, 12, 24 and 48 hours respectively. In Figure 4, it was found that AR began to inhibit the cell growth significantly at the 12th hour and the effect was also improved linearly while prolonging the incubation time.

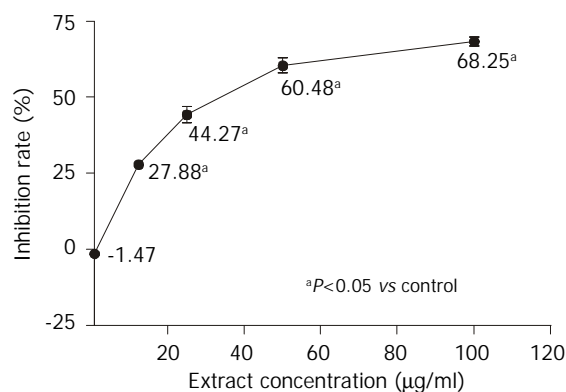


Figure 3 Dose-dependent effect of AR on AGS growth. AGS cells were treated with a serial of diluted concentrations (1, 12.5, 25, 50 and 100 $\mu\text{g}/\text{ml}$) of AR for 48 hrs and cell growth was measured with MTS method. The inhibition effect of AR was improved accompanied by increase of concentration.

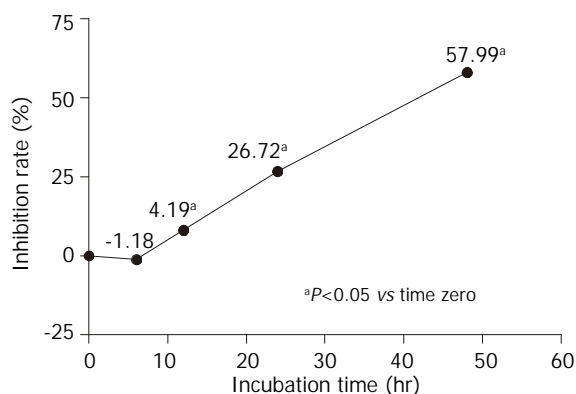


Figure 4 Time-dependent effect of AR on AGS growth. AGS cells were treated with 50 $\mu\text{g}/\text{ml}$ of AR for 6, 12, 24 and 48 hrs. The cell growth was measured by MTS method. The inhibition effect of AR on AGS growth was enhanced with incubation time.

Effect of AR on AGS DNA synthesis

Tritium-labeled thymidine incorporation assay was used to evaluate the effect of AR on AGS DNA synthesis. AGS cells were treated with different concentrations of AR for 48 hours. Figure 5 demonstrated that AR could significantly inhibit the DNA synthesis of AGS. It was also observed that the inhibiting effect of AR increased sharply from the concentrations of 1 $\mu\text{g}/\text{ml}$ to 25 $\mu\text{g}/\text{ml}$ and it increased slowly from then on.

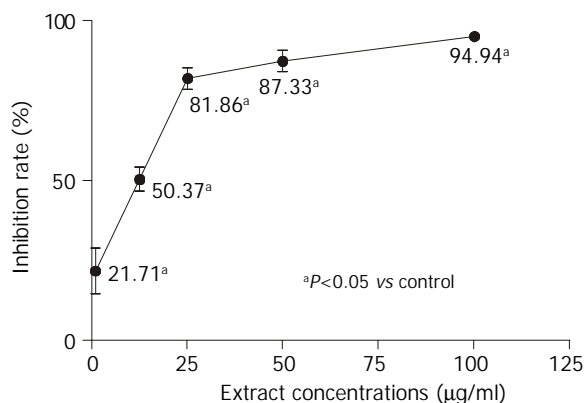


Figure 5 Effect of AR on DNA synthesis of AGS. AGS cells were treated with a serial of diluted concentrations (1, 12.5, 25, 50 and 100 $\mu\text{g}/\text{ml}$) of AR for 48 hrs and cell growth was measured with tritium labeled thymidine incorporation assay. AR started to inhibit the DNA synthesis of AGS at 1 $\mu\text{g}/\text{ml}$ and the inhibiting effect was dose-dependent.

Effect of AR on AGS apoptosis

DNA fragmentation method was used to observe whether apoptosis was induced in AGS by AR. AGS cells were treated with different concentrations of AR for 48 hours. However, there was no DNA ladder appeared in the electrophoresis gel. So the herbal extract had no effect on AGS apoptosis.

DISCUSSION

AR is a tonic herbal medicine which is used as an adjuvant extensively in the treatment of various cancers, such as lung cancer, digestive tract cancers, renal cancer, bladder cancer^[1,2,11], melanoma and AIDS^[12]. It had been reported that it could improve the host immune function suppressed by tumors and relieve the marrow suppression induced by chemotherapeutic agents so that it could make the patients more tolerant to chemotherapeutic therapy. Although there were some studies showing that AR could inhibit the tumor growth *in vivo*, the anti-cancer effect was testified through activating the activity of Th, LAK and macrophages and promoting the production

of IL-2 and IFN- γ . However, whether it has direct inhibition effect on tumors cells has not been elucidated yet.

In this study, 6 different cancer cell lines were used to test the inhibitory effect of AR. After 48 hours incubation, AR inhibited the gastric cancer cell lines of AGS and KATOIII growth by 68.25 % and 62.36 %, compared to the inhibition rates of colon cancer cell lines HT29, breast cancer cell line MDA231 and melanoma cell lines of Mel7 and Mel14 which were 22.8 %, 27.69 %, 2.85 % and 5.14 % respectively, suggesting that AR has anti-cancer effect on gastric cancer cell lines. To fully confirm this, many other kinds of cancer cell lines should be tested in order to determine the specificity of the effect. AGS and KATOIII are two different gastric cancer cell lines. AGS is a moderately-poorly differentiated adenocarcinoma cell line while KATOIII is a cell line of signet ring cell carcinoma, but AR has almost the same inhibitory effect on both cell lines.

Many herbal extracts can inhibit cell growth *in vitro* through their cytotoxic effect^[13-16]. We determined whether the inhibitory effect of AR was due to cytotoxicity. In order to clarify this question, we measured the cell growth with trypan blue exclusion method. The initial cell number was 300 000/well and in the untreated wells the cell number reached 990 000/well after 48 hours incubation. The cell number in testing group treated with 1 $\mu\text{g}/\text{ml}$ of AR was 943 500/well ($P>0.05$, vs control group). However, the cell numbers of the other testing groups treated with AR from the concentrations of 12.5 $\mu\text{g}/\text{ml}$ to 100 $\mu\text{g}/\text{ml}$ were significantly lower than that of the control group and but higher than the initial cell number except for the testing group treated with 100 $\mu\text{g}/\text{ml}$ AR, whose cell number was only 243 000/well ($P=0.017$, compared to the initial cell number). Few cells stained trypan blue could be seen in the testing groups treated with AR at the concentration lower than 100 $\mu\text{g}/\text{ml}$. So the inhibition effect of AR at the concentrations of 50 $\mu\text{g}/\text{ml}$ or below was not cytotoxic while AR is cytotoxic to AGS cell at the concentration of 100 $\mu\text{g}/\text{ml}$. Further studies showed that the inhibition effect of AR on AGS was dose- and time-dependent. The maximal concentration without cytotoxicity was around 50 $\mu\text{g}/\text{ml}$ and the corresponding inhibition rate was 60.48 %. AR came into effect at the 12th hour and reached its peak at the 48th hour.

One characteristic of the tumor cells is that their rapid proliferations are accompanied by DNA synthesis and cellular replication. Tritium labeled thymidine incorporation assay reflects the cellular DNA synthesis. Our results showed that the DNA synthesis of AGS was significantly inhibited by AR. The inhibition rate increased sharply from 21.71 % to 81.86 % at the concentrations ranging from 1 $\mu\text{g}/\text{ml}$ to 25 $\mu\text{g}/\text{ml}$, then it reached a plateau. So AR might suppress AGS cell growth through inhibition of DNA synthesis. The inhibition rates of AGS cell growth expressed by MTS method and tritium labeled thymidine incorporation assay were different. The latter was higher than the former. This may be attributed to that MTS and tritium labeled thymidine incorporation assay were two different indirect measurements used in cell growth evaluation. MTS reflects the cell growth at the level of cell respiration while tritium labeled thymidine incorporation assay at the level of DNA synthesis. When the cellular DNA synthesis is suppressed, the cells might be in an inactive status in which the cells could still respire. So the inhibition rate of cell growth expressed by MTS is lower than that expressed by tritium labeled thymidine incorporation assay.

Apoptosis is a kind of programming cell death whose impairment may induce cell immortality and carcinogenesis^[17]. Some agents such as aspirin, indomethacin and arsenic trioxide^[10,18] and some herbs such as Anemarrhena asphodeloides Bunge, Albizzia Lucidior I. Nielsen and Paeoniae radix^[19-21] were reported to have the ability of inducing apoptosis in gastric

cancer cells and inhibiting their growth. However, apoptosis could not be observed in AGS cells treated with AR. Combined with the results of trypan blue experiment that there were few cells, which were treated with AR, stained blue, the inhibition effect of AR on AGS is not through the mechanism of cytotoxic or apoptosis.

The aqueous and organic extracts of AR were used in this study. But the organic extract had no effect on AGS growth (data not shown). So the effective anti-cancer components are in the aqueous extract. Polysaccharide^[22], astragaloside^[23], and flavonoids^[24] have been found in AR. Some studies showed that the polysaccharides and flavonoids from other herbs had the anti-tumor efficiency *in vitro* and *in vivo*^[25-29]. But up to now, there has no evidence showing that these compounds from AR have anti-cancer activity *in vitro*. Hence, further studies are required. Selenium toxicity has been confirmed in the livestock consuming plants of the genus *Astragalus* and selenium was reported to have the carcinostatic activity in the animals^[30,31]. So selenium might play a critical role in AR inhibiting AGS growth.

In conclusion, the inhibitory effect of AR on the growth of gastric cancer cell line of AGS is mainly cytostatic.

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