Effects of aristolochic acid on morphological transformation of cultured Syrian hamster embryo cells

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Abstract: **AIM** A large body of evidence has shown that transformation of Syrian hamster embryo (SHE) cell cultures is perhaps the most biologically relevant short-term system for identifying carcinogens and studying the mechanisms of chemical carcinogenesis. The purpose of the present study is to examine the ability of aristolochic acid (AA), the active component of many herbal medicines derived from *Aristolochia* to induce morphological transformation (MT) in cultured SHE cells. In addition, the effect of α-tocopherol on AA-induced MT was studied. **METHODS** Prior to the transformation assay, a dose range-finding study was conducted employing a wide range of concentrations of AA following either a 24 h or a 7 d treatment to establish an appropriate range of concentrations for the cell transformation assay. AA concentrations causing approximately 0% – 50% cytotoxicity were chosen for testing in the cell transformation assay. The effect of α-tocopherol on AA-induced MT was investigated by co-treatment of the cultured SHE cells with transforming concentrations of AA and 100 $\mu mol \cdot L^{-1}$ α-tocopherol. **RESULTS** Following a 7 d continuous treatment, AA induced significant increases in MT at concentrations of 0.4, 0.8 and 1.6 $mg \cdot L^{-1}$ (maximum subtoxic concentration tested). Significant increases in MT were also observed in SHE cells treated with AA at concentrations of 1.0, 1.5, 2.5, 4.5 and 5.0 $mg \cdot L^{-1}$ (maximum subtoxic concentration tested) for 24 h. Transformation induced by AA was inhibited (16% – 76%) by co-treatment with the antioxidant, α-tocopherol (100 $\mu mol \cdot L^{-1}$). **CONCLUSION** The results of the present study show that AA can induce MT in cultured SHE cells following either a 24 h treatment or a 7 d continuous treatment. MT induced by AA can be inhibited by antioxidant, α-tocopherol, suggesting that oxidative stress be involved in AA-induced transformation and carcinogenesis. The present study also shows that the SHE cell transformation assay can be a useful tool for the nutraceutical and herbal medicine industry to detect potential carcinogenic ingredients as well as to screen potential anticarcinogenic ingredients in their products.

Key words: aristolochic acid; cell transformation, neoplastic; embryo, mesocricetus; vitamin E

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Herbs have been used for thousands of years as medicines to treat diseases[1]. Most of them are used as medicines with little understanding of their potential toxicological effects. *Aristolochia* is a typical example of these herbal medicines[2]. The *Aristolochia* family is one of the oldest plant species used as herbal medicine, with aristolochic acid being the major active component[3]. This family of herbal medicines has been used for many years to treat certain diseases such as arthritis, gout, rheumatism, and snake bites[4,5]. Aristolochic acid (AA) can be isolated from these plants as a mixture of two structurally related phenanthrene carboxylic acids (AA Ⅰ and AA Ⅱ), which differ only by one methoxy group[6]. Besides the above therapeutic effects, AA was also shown to have antifungal, antibacterial, antiviral, and antitumor effects[7-10]. Therefore, AA was used as an anti-inflammatory agent in several pharmaceutical preparations prior to 1982. However, two independent studies in the early 1980′s showed that AA was a potent rodent carcinogen[11,12]. As a result, the products containing AA were withdrawn from the market by the German Federal Health Office. Although the therapeutic claims of herbs are not allowed in the United States, the Dietary Supplement Health and

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Education Act (DSHEA) passed in 1994 allows herbs and other botanicals be used as dietary supplements. Contamination with AA was recently found in 18 of the 35 dietary supplement products tested by the US FDA[13]. In order to protect consumers from the potential health risks related to AA, the US FDA requested manufacturers and distributors to recall all botanical products containing AA.

After the carcinogenic effects of AA were reported, many studies were conducted to investigate the genotoxicity of AA. AA was shown to be a direct mutagen in *Salmonella typhimurium* tester strains TA100, TA102 and TA1537[14,15], but AA was not mutagenic in the nitroreductase-deficient strain TA100NR[14]. AA was also shown to be mutagenic to cultured Chinese hamster ovary (CHO) cells[16]. In addition, induction of structural chromosomal aberrations and micronuclei in human lymphocytes by AA was reported[17,18].

Morphological transformation (MT) of Syrian hamster embryo (SHE) cells by chemical carcinogens was first reported by Berwald and Sachs[19] in the early 1960′s. A large body of evidence has shown that SHE cell transformation is one of the most predictive *in vitro* assays for identifying carcinogens. The major difference between the SHE cell transformation assay and commonly used short term genetic toxicology assays is that the end point of this assay, MT, is the preneoplastic conversion of cells *in vitro*, which is very similar to the preneoplastic or neoplastic alteration of cells *in vivo*[20]. Therefore, MT can be induced by both genotoxic and nongenotoxic carcinogens. In contrast, most of the commonly used short term genetic toxicology assays only respond to genotoxic carcinogens. With more than 500 chemical/physical agents tested in this system, the SHE cell transformation assay demonstrates more than 80% concordance with the results of rodent bioassays[21]. In addition, SHE cells demonstrate a multistage process of neoplastic transformation similar to that observed *in vivo*[22,23]. Transformation of SHE cells may result from similar mechanisms involved in carcinogenic process *in vivo*. Therefore, this transformation system not only provides a useful system to evaluate the carcinogenic potential of chemical/physical agents, it also provides a relevant model to study the mechanisms of carcinogenesis *in vitro*[24]. Besides evaluating the carcinogenic potential of chemicals and studying the mechanisms of carcinogenesis, the SHE cell transformation system has also been used to assess the chemopreventive potential of chemicals and to investigate their mechanisms of action[25,26]. In the present study, the potential of AA to induce MT and the ability of α-tocopherol, a well-known antioxidant, to inhibit AA-induced MT were studied in the SHE cell transformation system.

1　MATERIALS AND METHODS

1.1　Chemicals

Aristolochic acid was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Benzo(a)pyrene (BaP), dimethylsulfoxide (DMSO), α-tocopherol acetate, and *L*-glutamine were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from HyClone (Logan, UT). LeBoeuf′s modification of DMEM (DMEM-*L*) was obtained from Quality Biological (Gaithersburg, MD).

1.2　SHE cell transformation assay

SHE cell transformation was conducted as described previously by Custer, *et al*[27]. Briefly, primary embryo cells were isolated from 13-d embryos of Syrian hamsters and cryopreserved. Each experiment began with cells taken from the cryopreserved lot and cultured to achieve near-confluency, followed by seeding the cells into the assay dishes. The cell cultures for the SHE assay contained 40 000 cells/culture that had been X-irradiated sufficiently to prevent division but not metabolism (called "feeder cells") and a smaller number of untreated cells to act as "targets" for the test chemical. The feeder cells were seeded first, and the target cells (enough to produce 25 – 45 colonies/dish) were seeded a day later. After incubation for another day, the cultures were exposed to AA either for 24 h (followed

by replacement of the medium with AA-free medium for an additional 6 d) or for 7 d continuously. In studies examining the effects of α-tocopherol, AA and α-tocopherol(100 μmol·L^{-1}) were co-incubated for either 24 h or 7 d. After incubation, the cultures were fixed with methanol, stained with Giemsa, and evaluated for morphological transformation.

Each SHE cell transformation assay was preceded with a dose range-finding assay in which a wide range of chemical concentrations was tested for cytotoxicity. After incubation, the reduction in colony number(or colony density) was determined relative to the vehicle control cultures. Chemical concentrations causing approximately 0% - 50% reduction in colony number or density were chosen for testing in the cell transformation assay.

1.3 Statistic analysis

Data were analyzed using Fisher's exact test.

2 RESULTS

2.1 Cytotoxicity of aristolochic acid

Initial studies were performed to examine the cytotoxicity of AA(measured by reduction of relative plating efficiency, RPE) in SHE cells after either a 7 d continuous treatment or a 24 h treatment. A concentration range of 0.025 to 50 mg·L^{-1} was tested for the 7 d continuous treatment. A dose dependent increase in cytotoxicity was produced by AA, and substantial cytotoxicity (> 50% reduction of RPE) was observed at doses of 3.14 mg·L^{-1} and greater(Tab 1). AA was lethal at 12.5 mg·L^{-1}. Similarly, a range of 0.20 to 100 mg·L^{-1} was tested for the 24 h treatment period. Substantial cytotoxicity was obtained at concentration of 6.25 mg·L^{-1} and above, and AA was also essentially lethal at 12.5 mg·L^{-1}(Tab 1).

Tab 1. Cytotoxicity of aristolochic acid(AA) in Syrian hamster embryo(SHE) cells following 7 d or 24 h treatments

7 d Treatment				24 h Treatment			
Treatment	Average number colonies per dish	PE /%	RPE /%	Treatment	Average number colonies per dish	PE /%	RPE /%
DMSO 0.2%	31	19.1 ± 2.1	100	DMSO 0.2%	22	19.1 ± 2.1	100
AA(mg·L^{-1})				AA(mg·L^{-1})			
0.025	28	17.3 ± 2.8	91	0.200	22	17.3 ± 2.8	104
0.050	28	17.8 ± 2.4	93	0.400	19	17.8 ± 2.4	87
0.100	27	17.1 ± 2.7	90	0.800	23	17.1 ± 2.7	105
0.200	29	18.3 ± 3.8	96	1.60	19	18.3 ± 3.8	87
0.400	23	14.5 ± 1.9	76	3.14	13	14.5 ± 1.9	60
0.800	21	13.4 ± 3.1	70	6.25	6	13.4 ± 3.1	27
1.6	17	5.90 ± 1.4	55	12.5	0.2	5.90 ± 1.4	1
3.14	9	2.20 ± 0.9	31	25.1	0	2.20 ± 0.9	0
6.25	4	0	12	50.0	0	0	0
12.5	0	0	0	100	0	0	0
25.0	0	0	0	–	–	–	–
50.0	0	0	0	–	–	–	–

PE: plating efficiency = (number of colonies per dish/number of target cells seeded) × 100%. RPE: relative plating efficiency = (PE of treatment group/PE of vehicle control group) × 100%. $\bar{x} \pm s$.

2.2 Morphological transformation by aristolochic acid

Based on the results of the cytotoxicity studies, five concentrations of AA ranging from 0.20 to 1.6 mg·L^{-1} were tested in the 7 d transformation assay. An RPE range of 88% to 42% cytotoxicity range of 12% to 58% was caused by the AA treatments in this concentration range (Tab 2). Significant increases in the frequency of MT were observed at concentrations of 0.40, 0.80, and 1.6 mg·L^{-1}. When SHE cells were treated with AA at concentrations from 1.0 to 5.0 mg·L^{-1} for 24 h, a concentration-dependent increase in cytotoxicity was produced, and significant increases in MT frequency were obtained from all treatment groups except for the 3.5 mg·L^{-1} treatment (Tab 2). For both exposure conditions, the positive control treatment with BaP at 5.0 mg·L^{-1} induced a significant, and similar, increase in transformation frequency compared to the concurrent vehicle control (Tab 2).

2.3 Effect of α-tocopherol on aristolochic acid-induced morphological transformation

The results from the transformation assay were used to select 0.40 and 0.80 mg·L^{-1} of AA (as transforming treatments) to test in combination with α-tocopherol. As shown in Tab 3, treatment with either 0.40 or 0.80 mg·L^{-1} AA for 7 d produced significant increases in MT frequency, as expected. However, co-incubation with 100 μmol·L^{-1} α-tocopherol reduced the MT frequency; the cellular transformation induced by 0.40 mg·L^{-1} and 0.80 mg·L^{-1} AA was inhibited by 56% and by 16%, respectively. This inhibition was statistically significant for the co-treatments with 0.40 mg·L^{-1} AA and 100 μmol·L^{-1} α-tocopherol, relative to AA alone (Tab 3). Similar results were obtained for a 24 h treatment period. MT induced by 1.0 mg·L^{-1} AA was significantly inhibited (by 76%) by co-treatment with 100 μmol·L^{-1} α-tocopherol (Tab 4). The data also clearly showed that treatment with 100 μmol·L^{-1} α-tocopherol alone did not have any effect on MT in SHE cells for either the 7 d or 24 h exposure periods.

3 DISCUSSION

The SHE cell transformation assay has been used for decades to evaluate the carcinogenic potential of chemicals[28]. Two protocols are currently in use for the SHE cell transformation

Tab 2. AA-induced morphological transformation in SHE cells following 7 d or 24 h treatments

7 d Treatment					24 h Treatment				
Treatment	MTF /%	Total MT colonies	Total colonies	RPE /%	Treatment	MTF /%	Total MT colonies	Total colonies	RPE/%
DMSO 0.2%	0.280	3	1073	100	DMSO 0.2%	0.377	4	1060	100
BaP 5.0 mg·L^{-1}	2.255*	26	1153	110	BaP 5.0 mg·L^{-1}	1.964*	22	1120	105
AA(mg·L^{-1})					AA(mg·L^{-1})				
0.20	0.528	5	947	88	1.00	1.189*	22	757	71
0.40	1.316*	12	912	85	1.50	1.480*	11	743	70
0.80[a]	1.489*	13	873	57	2.50	1.351*	11	814	55
1.20[a]	0.564	5	886	49	3.50	0.841	7	832	47
1.60[a]	1.217*	11	904	42	4.50	1.939*	14	722	35
–	–	–	–	–	5.00	1.733*	14	808	31

BaP: benzo(a) pyrene. MTF: morphological transformation frequency = (number of transformed colonies/total colonies) × 100%. a: The number of target cells seeded per dish was increased to compensate for expected toxicity. * $P \leqslant 0.05$, compared to the concurrent vehicle control by Fisher's exact test.

Tab 3. Inhibition of AA-induced transformation by α-tocopherol following 7 d treatment

Treatment	MTF/%	Total MT colonies	Total colonies	RPE/%
Vehicle	0.283	3	1059	100
BaP 5.0 mg·L^{-1}	1.991*	23	1155	106
AA 0.40 mg·L^{-1}	1.393*	13	933	88
aAA 0.80 mg·L^{-1}	1.169*	13	1112	71
α-Tocopherol 100 μmol·L^{-1}	0.353	4	1134	107
AA 0.40 mg·L^{-1} + α-tocopherol 100 μmol·L^{-1}	0.614#	6	977	90
aAA 0.80 mg·L^{-1} + α-tocopherol 100 μmol·L^{-1}	0.987	11	1114	71

See Tab 2 for the legend. * $P \leqslant 0.05$, compared with concurrent vehicle control by Fisher's exact test; # $P \leqslant 0.05$, compared with corresponding AA group by Fisher's exact test.

Tab 4. Inhibition of AA-induced transformation by α-tocopherol following 24 h treatment

Treatment	MTF/%	Total MT colonies	Total colonies	RPE/%
Vehicle	0.331	4	1209	100
BaP 5.0 mg·L^{-1}	1.424*	21	1475	122
AA 1.00 mg·L^{-1}	1.955*	20	1023	85
α-Tocopherol 100 μmol·L^{-1}	0.150	2	1335	110
AA 1.00 mg·L^{-1} + α-tocopherol 100 μmol·L^{-1}	0.472#	6	1271	105

See Tab 2 for the legend. * $P \leqslant 0.05$, compared with concurrent vehicle control by Fisher's exact test; # $P \leqslant 0.05$, compared with corresponding AA group by Fisher's exact test.

assay: one using a 7 d exposure period, and the other, a 24 h exposure. The results from previous studies have shown that treatment duration-dependent transformation was indicative of the potential mode of action for compounds[29]. A negative result with a 24 h treatment, in combination with a positive result from a 7 d treatment, may be indicative of a "promotion-like" activity of a test compound. For initiator type of compounds, positive results are usually obtained with both treatment protocols. Results from the present study showed that AA induced a significant increase in MT in SHE cells following either a 7 d or 24 h exposure. These results support an initiation mode of action (direct interaction with DNA) for AA-induced transformation. In fact, previous studies by others have clearly shown that AA is a direct DNA damaging agent, which produces gene mutations[14-16,30], structural chromosome aberrations[17], and unscheduled DNA synthesis[31] in different *in vitro* and *in vivo* systems. Taken together, the current SHE assay results and published genotoxicity assay results are consistent in indicating that AA is a genotoxic carcinogen.

The inhibition of AA-induced MT by co-treatment with α-tocopherol is an interesting finding from the current study. Previous studies showed that α-tocopherol inhibited MT induced by acrylonitrile by preventing formation of 8-hydroxy-2′-deoxyguanosine (OH8dG), the most predominant DNA oxidative modification[26]. Therefore, the inhibitory effects of α-tocopherol suggest a role for oxidative stress in the induction of MT by AA. α-tocopherol is one of four tocopherols composing the well-known, naturally occurring, fat-soluble antioxidant, vitamin E.

α-Tocopherol is the most abundant and the most biologically active component of vitamin E[32]. Within cells, α-tocopherol is mainly located in the phospholipid bilayer membrane, protecting the adjacent membrane lipids, unsaturated fatty acids, and proteins against oxidative damage caused by free radicals and blocking the free radical peroxidation process[33,34]. Through similar mechanisms, α-tocopherol can also prevent DNA from being damaged by oxidative stress[35]. However, other mechanisms, such as blocking the formation of active DNA-reacting metabolites[36], are also involved in the protective effects of α-tocopherol against DNA damage. In addition, since the inhibitory effects of α-tocopherol on AA-induced MT were observed *in vitro* under co-treatment condition, AA may be inactivated by direct reaction with α-tocopherol. Therefore, without further investigation, the actual mechanisms by which α-tocopherol inhibits MT induced by AA will remain unknown.

In summary, the results from the present study show that the rodent carcinogen, AA, was effective in causing MT in SHE cells, and furthermore, that the common antioxidant, α-tocopherol, decreased the MT induced by AA. AA may even

be preventive of AA-induced transformation for different combinations of AA and α-tocopherol concentrations used in this study. Both AA and α-tocopherol are components of many natural products. Therefore, the results from the current study suggest that SHE cell transformation be a useful model assay system for the nutraceutical and herbal medicine industries to detect potential carcinogenic ingredients as well as to screen for potential anticarcinogenic properties in their products.

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马兜铃酸对培养的叙利亚仓鼠胚胎细胞形态学转化的影响

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摘要: 目的　大量的研究证据表明,叙利亚仓鼠胚胎(SHE)细胞转化试验可能是目前用于检测致癌物和研究化学致癌机制的试验中生物相关性最强短期试验。本研究旨在检测来源于马兜铃鼠属类草药中的主要活性成分马兜铃酸(AA)引起 SHE 细胞形态学转化的可能性和抗氧化剂 α-生育酚对 AA 所诱导的 SHE 细胞形态学转化的影响,并以此为例来说明 SHE 细胞转化试验可以用于检测营养保健品和中草药中可能存在的致癌或抗癌成分。**方法**　在进行正式的细胞形态学转化试验之前,先进行了初步的剂量范围选择试验,以确定用于形态学转化试验的 AA 的浓度。剂量范围选择试验是在 24 h 染毒或 7 d 连续性染毒的两种条件下进行的。根据剂量范围选择试验的结果,那些引起 0% ~ 50% 细胞毒性的剂量被用于 24 h 染毒或 7 d 连续性染毒的细胞形态学转化试验来检测 AA 引起 SHE 细胞形态学转化的可能性。至于 α-生育酚对 AA 所诱导的 SHE 细胞形态学转化的影响,则使用了用可引起 SHE 细胞形态学转化浓度的 AA 和 100 $\mu mol \cdot L^{-1}$ α-生育酚同时处理 SHE 细胞的方法进行了观察。**结果**　在 7 d 连续性染毒的条件下,0.4, 0.8 以及 1.6 $mg \cdot L^{-1}$的 AA 引起了 SHE 细胞形态学转化率的显著性升高;在 24 h 染毒的条件下,1.0, 1.5, 2.5, 4.5 和 5.0 $mg \cdot L^{-1}$等 5 个剂量组的形态学转化率明显地高于阴性对照组。当在培养液中加入 100 $\mu mol \cdot L^{-1}$ α-生育酚后,AA 诱导的 SHE 细胞形态学转化则被抑制了 16% ~ 76%。**结论**　本研究的结果表明,无论在 24 h 染毒还是 7 d 连续性染毒的条件下,AA 都可以引起培养的 SHE 细胞的形态学转化。抗氧化剂 α-生育酚可以抑制这种由 AA 引起的形态学转化。此结果提示,氧化损伤可能是 AA 引起细胞转化和致癌作用的机制之一。本研究的结果也表明,SHE 细胞形态学转化试验对于研究开发营养保健品和中草药的机构来说是一个非常有用的工具。它可以用来检测这些产品中可能存在的致癌成分,也可以用来筛选产品中可能存在的抗癌成分。

关键词: 马兜铃酸; 细胞转化, 肿瘤; 胚胎, 金仓鼠; 维生素 E

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