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Growth Inhibition of A549 Human Lung Adenocarcinoma Cells by L-Canavanine Is Associated with p21/WAF1 Induction

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L-Canavanine (CAV) is a higher plant nonprotein amino acid and a potent L-arginine antimetabolite. CAV can inhibit the proliferation of tumor cells *in vitro* and *in vivo*, but little is known regarding the molecular mechanisms mediating these effects. We demonstrated that the treatment of human lung adenocarcinoma A549 cells with CAV caused growth inhibition; G1 phase arrest is accompanied by accumulation of an incompletely phosphorylated form of the retinoblastoma protein, whose phosphorylation is necessary for cell cycle progression from G1 to S phase. In addition, CAV induces the expression of p53 and subsequent expression of a cyclin-dependent kinase inhibitor, p21/WAF1. The p53-dependent induction of p21/WAF1 and the following dephosphorylation of the retinoblastoma protein by CAV could account for the observed CAV-mediated G1 phase arrest.

Key words: L-Canavanine — p21/WAF1 — p53 — RB — Cell cycle

L-Canavanine (CAV) [L-2-amino-4-(guanidinooxy)-butyric acid] is synthesized in the seeds of the legume, jack bean, *Canavalia ensiformis* (L.) DC. [Fabaceae].[1] This potent arginine antimetabolite is an important nitrogen-storing compound synthesized by many leguminous plants.[2]

CAV, a structural analogue of arginine, is a substrate for arginyl-tRNA synthetase[3] and is incorporated readily into proteins in place of arginine.[4] In biological systems, CAV incorporation produces structurally aberrant 'canavanyl' proteins which exhibit altered protein conformation and impaired function.[5,6] The analogue-substituted proteins are degraded more rapidly than their normal counterparts.[7–11] In addition, CAV is an inhibitor of inducible nitric oxide synthase.[12,13]

Antineoplastic properties of CAV have been reported. CAV inhibited the *in vitro* growth of human pancreatic cancer[14] and human melanoma.[15] This nonprotein amino acid sensitized human colon tumor cells to γ-irradiation[16] and enhanced the effect of 5-fluorouracil.[17] CAV attenuated *in vivo* growth of a rat colonic tumor[18] and a hemangioma transplanted into mice,[19] and prolonged the life of L1210 leukemia-bearing mice.[20] These studies established that CAV exhibits anti-tumor properties, but its mechanism of action is unknown.

Cell cycle regulators such as cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors are key factors for cell growth. G1 to S phase-transition is a major checkpoint in the cell cycle progression, and deregulation of the G1/S checkpoint is considered to be an important cause of carcinogenesis. The well-characterized CDK-inhibitory protein p21/WAF1 is believed to exert its growth-inhibitory effect primarily during G1 phase, through binding to multiple CDK[21–24] and suppressing the phosphorylation of the retinoblastoma protein (pRB) in a dose-dependent fashion.[25] In many mammalian cell lines, transcription of the *p21/WAF1* gene is known to be directly promoted by wild-type *p53*.[26]

In this study, we examined the effect of CAV on the human lung adenocarcinoma A549 cell line. CAV treatment caused growth inhibition in A549 cells associated with G1 phase arrest accompanied by the accumulation of an incompletely phosphorylated form of pRB. To investigate further the molecular mechanisms of the G1 phase arrest by CAV, we examined the influence of this drug on the factors involved in regulating G1 progression. We found a correlation between p53 protein induction and p21/WAF1 expression in CAV-treated A549 cells. These data suggest that the growth inhibition of A549 cells by CAV could be due to the p53-dependent induction of p21/WAF1 and subsequent suppression of the phosphorylation of pRB.

MATERIALS AND METHODS

Drug and cell culture CAV was isolated and purified as described elsewhere (Fig. 1).[27] It was dissolved in sterilized water and diluted to the appropriate concentration. A549 cells were maintained in Dulbecco's modified

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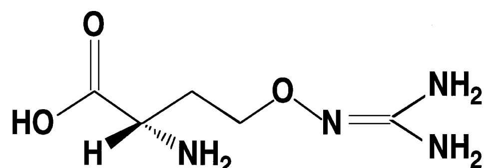

Fig. 1. Structure of L-canavanine (CAV).

Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. A549 cells harbor wild-type *p53*[28] and *RB*[29] genes, and lack a functional *p16* gene.[29]

Growth inhibition of cells The initial cell density was 4×10^4 cells/2 ml medium in 35-mm diameter dishes. CAV was added at various concentrations on the second day after inoculation. On days 3 and 4, the number of viable cells was determined by a Trypan-blue dye exclusion test. This cell-growth study was carried out in triplicate and repeated three times.

Analysis of cell cycle progression Cells were plated at a density of 2×10^5 cells/5 ml medium in 100-mm diameter dishes. Two days after inoculation, CAV was added. Twenty-four and 48 h after the addition of the drug, cells were removed from the culture dishes by trypsinization. The trypsinized cells were suspended in 5 ml of complete medium and collected by centrifugation (150*g*). The cells were washed with 5 ml of phosphate-buffered saline (PBS) and suspended in 1 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a 50 μM nylon mesh, then adjusted to a final concentration of 0.1% (w/v) RNase and 50 μg/μl propidium iodide. The DNA content of the stained nuclei was analyzed with a FACScan (Becton Dickinson). A DNA histogram was prepared for each cell suspension sample. The number of stained nuclei in each phase was measured by use of the S-fit program in the FACScan.

RNA isolation and northern blot analysis Cells were inoculated at a density of 2.5×10^5 cells/5 ml medium in 100-mm diameter dishes. Two days after the inoculation, CAV was added at various concentrations. At the indicated times, the total RNA was isolated from these cells using a TRIzol RNA isolation kit (GIBCO BRL); 10 μg of total RNA per lane was examined by northern blot analysis. The p21/WAF1 cDNA for the probe was obtained from pCEP-WAF1 plasmid (a kind gift from Dr. B. Vogelstein), by digesting with *Not*I. Northern blot analysis was performed by a standard method.[30] A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control. The mRNA level was determined using a bioimaging analyzer, BAS 2000 (Fujix).

Protein isolation and western blot analysis The cells were inoculated at a density of 2.5×10^5 cells/5 ml medium in 100-mm diameter dishes. Two days after the inoculation, CAV was added at the indicated concentrations. At the indicated times, the cell lysate was prepared as follows. Cells were washed twice with 8 ml of PBS, and the sample buffer [10% glycerol (v/v), 1% sodium dodecyl sulfate (SDS) (w/v), 5% 2-mercaptoethanol (v/v), 50 m*M* Tris-HCl (pH 6.8), 0.025% bromophenol blue (w/v)] was directly added to the cells. The loading sample buffer's volume was normalized according to the cell number of each dish so as to load a comparable amount of protein on each lane. The lysate was solubilized by boiling for 5 min and the proteins were fractionated by electrophoresis in 8% (w/v) (for RB detection) or 12% (w/v) (for p21/WAF1, p27 and p53 detection) SDS-polyacrylamide gel. After 1 h of electrophoresis at 20 mA, the proteins were transferred to a nitrocellulose filter (Schleicher & Schuell) under semi-dry conditions. The filter was soaked for 3 h in blocking buffer [4% skim milk (v/v, DIFCO), and 0.2% Tween-20 (v/v) in PBS] at 25°C. Afterwards the filter was incubated overnight at 4°C with anti-RB human monoclonal antibody (PM-14001A from Pharmingen, 1:500 dilution) or anti-p21/WAF1 human monoclonal antibody (PM-15091A from Pharmingen, 1:500 dilution), anti-p27 human monoclonal antibody (PM-13231A from Pharmin-

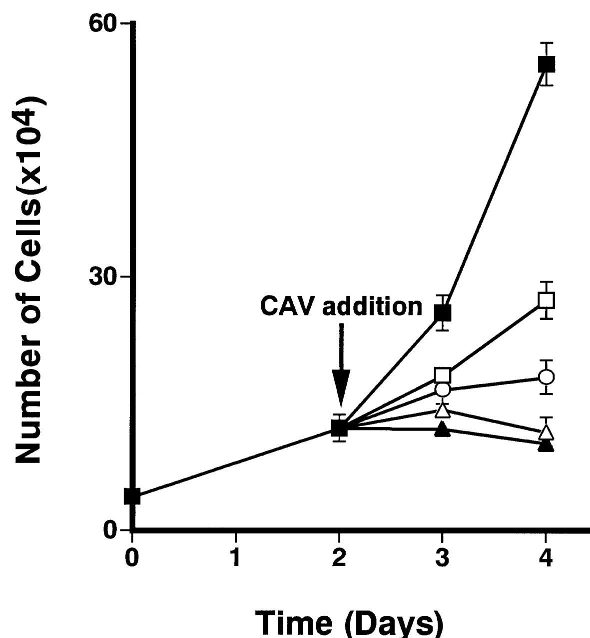

Fig. 2. Effects of CAV on the growth of A549 cells. Two days after cell inoculation, CAV was added at 0.5 (□), 1 (○), 2 (△), and 3 (▲) m*M* compared with control culture (■). On days 3 and 4, viable cell number was determined by a Trypan-blue dye exclusion test. Data are shown as means±SD (n=3).

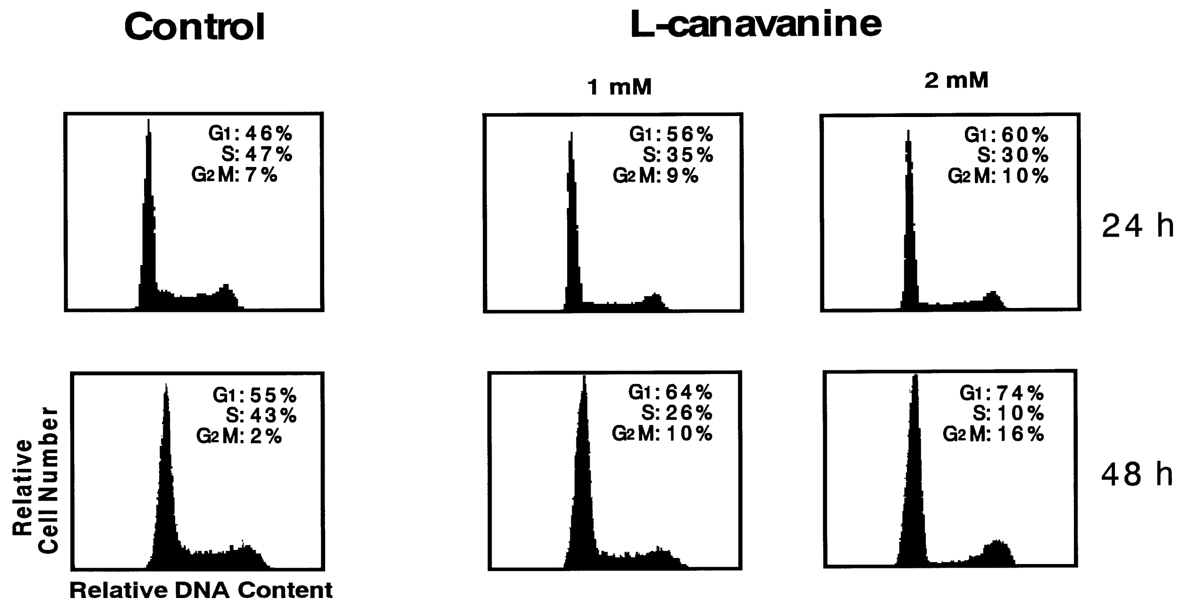

Fig. 3. Effect of CAV on the cell cycle progression in A549 cells. Two days after the inoculation, 1 or 2 m*M* CAV was added as indicated. At 24 or 48 h after the addition, DNA histograms of cells were obtained by flow-cytometric analysis. The percentage of each phase was calculated by S-fit analysis.

gen, 1:500 dilution), and anti-p53 human monoclonal antibody used at 1:1 dilution (anti-p53 human monoclonal antibody was a generous gift from Drs. K. H. Vousden and S. Bates, Maryland).

On the following day, the filter was washed with 0.2% (v/v) Tween-20 in PBS for 20 min and incubated with anti-mouse horseradish peroxidase conjugated with IgG (Amersham, 1:1000 dilution) for 1 h at 25°C. The filter was washed with 0.2% Tween-20 in PBS for 20 min and with PBS for 10 min at 25°C, and the proteins of interest were detected by enhanced chemiluminescence (ECL, Amersham).

RESULTS

CAV arrests A549 cells at G1 phase in cell cycle First, we examined the effect of CAV on A549 cell proliferation. Fig. 2 reveals the growth curve of A549 cells exposed to the indicated concentration of CAV. A dose-dependent inhibition of A549 cell growth was observed between 0.5 and 3 m*M*. On day 4, cell growth diminished to 50, 33, 21 and 18.6% of the control level with 0.5, 1, 2 and 3 m*M* CAV, respectively.

To investigate the effect of CAV on A549 cell cycle progression, the DNA content of the A549 nuclei was measured by flow-cytometric analysis 24 and 48 h after CAV addition. As shown in Fig. 3, the DNA histograms revealed that a 24 or 48 h exposure to 1 or 2 m*M* CAV decreased the S phase population of A549 cells. Consistent with the DNA histograms, S-fit analysis revealed that 2 m*M* CAV caused an enhanced accumulation of G1 phase cells from 46 to 60% (24 h) or from 55 to 74% (48

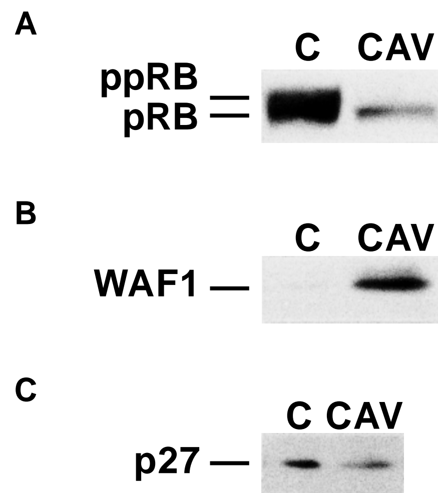

Fig. 4. Western blot analysis of RB, p21/WAF1 and p27 protein in A549 cells treated with or without CAV. A549 cells were exposed either to medium alone (C) or to medium containing 2 m*M* CAV (CAV) for 48 h, and then the expression of RB (A, upper panel), p21/WAF1 (B, middle panel) and p27 (C, lower panel) proteins was analyzed.

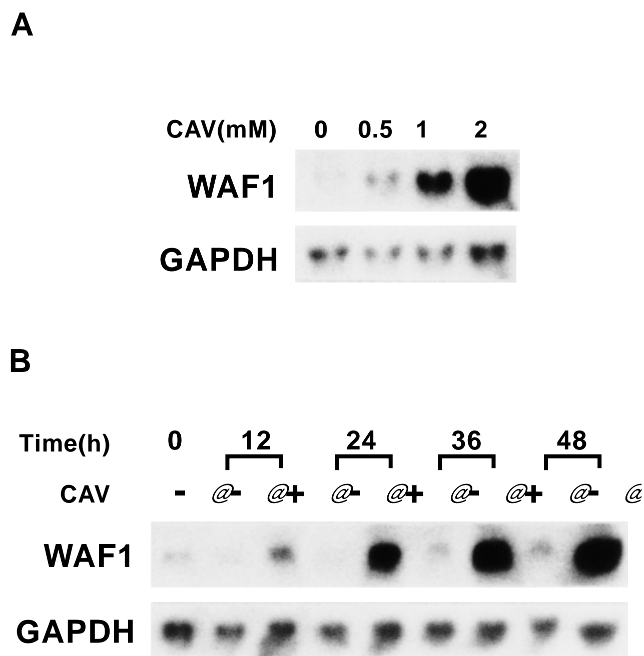

Fig. 5. Northern blot analysis of p21/WAF1 mRNA in A549 cells. A, A549 cells were treated with various concentrations of CAV (0, 0.5, 1, 2 m*M*). The expression of mRNA was examined 48 h later. B, A549 cells were exposed either to medium alone (–) or to 2 m*M* CAV (+), and total RNA was extracted at the indicated times after the addition of CAV. The same blot was hybridized with a GAPDH probe to normalize the amount of RNA loaded.

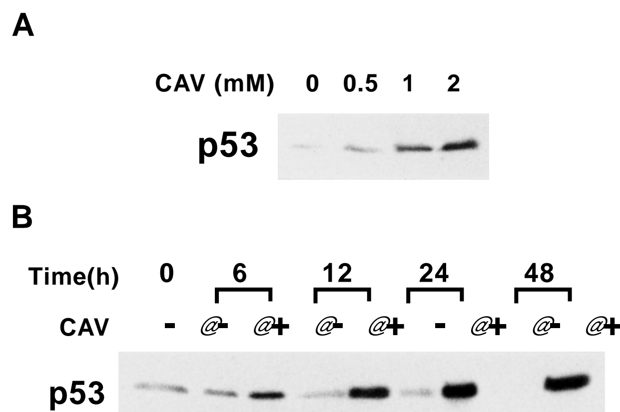

Fig. 6. Western blot analysis of p53 protein in A549 cells. A, A549 cells were treated with various concentrations of CAV (0, 0.5, 1, 2 m*M*). The level of p53 protein was examined 36 h after exposure to CAV. B, A549 cells were exposed either to medium alone (–) or to 2 m*M* CAV (+), and total protein was obtained at the indicated times after the addition of CAV, then the expression of p53 protein was analyzed.

h) while attenuating S phase cells from 47 to 30% (24 h) or from 43 to 10% (48 h).

We also examined the effect of CAV on p53-inactivated cells, such as a human bladder cancer cell line HTB9, and a human cervical cancer cell line HeLa. After a 48 h exposure to CAV, the cell growth diminished to 89, 57, 34 and 26% (HeLa) or 70, 72, 26 and 22% (HTB9) of the control level with 0.5, 1, 2 and 3 m*M* CAV, respectively (data not shown). Although CAV inhibited the proliferation of HeLa and HTB9 cells, we detected no G1 phase arrest by addition of 2 m*M* CAV (data not shown).

CAV causes dephosphorylation of RB protein To investigate the mechanism of CAV-mediated G1 phase arrest, we examined the phosphorylation status of pRB, since the phosphorylation of pRB mediated by CDK is required for cell cycle progression at the G1 to S phase transition. As shown in Fig. 4A, a 48 h exposure to this drug changed pRB from a mostly phosphorylated to a completely dephosphorylation form. This result suggested that the G1 phase arrest mediated by CAV could be due to failed pRB phosphorylation.

CAV increases p21/WAF1 mRNA level The most likely explanation for the observed inhibition of pRB phosphorylation could be the induction of CDK inhibitor(s) by CAV. To investigate whether the general CDK inhibitor, p21/WAF1 mRNA, is induced by CAV, A549 cells treated with CAV as well as control cells were subjected to northern blot analysis. As the data of Fig. 5 demonstrate, CAV induced p21/WAF1 mRNA expression in A549 cells. In the control cells, p21/WAF1 expression was weak, but a 48 h exposure to 0.5 to 2 m*M* CAV caused a dose-dependent induction of p21/WAF1 (Fig. 5A). Consistent with the above findings, CAV arrested cell cycle progression in a dose-dependent manner. The time course study disclosed that p21/WAF1 mRNA was initially induced around 12 h after treatment with 2 m*M* CAV, and reached its peak at 48 h. The difference at the 48 h point in the p21/WAF1 expression level between the CAV-treated and the control cells reached nearly 8.5-fold (Fig. 5B).

CAV induces p21/WAF1 protein To elucidate whether the p21/WAF1 protein is also induced by CAV, the appropriate cells were assayed for p21/WAF1 protein expression by western blot analysis. As revealed in Fig. 4B, treatment with 2 m*M* CAV for 48 h markedly enhanced p21/WAF1 protein expression. In contrast, another general CDK inhibitor, p27, was not induced when A549 cells were exposed to 2 m*M* CAV for 48 h (Fig. 4C). In p53-inactivated cell lines HTB9 or HeLa, the induction of p21/WAF1 mRNA was not observed (data not shown). Also, cell cycle arrest at G1 phase was not detected (data not shown). This result indicated that p21/WAF1 protein was specifically induced by CAV and was consistent with its induction of p21/WAF1 mRNA.

p21/WAF1 induction is preceded by p53 induction by CAV treatment Since the induction of p21/WAF1 is often preceded by the induction of p53 protein, we examined whether p21/WAF1 induction required the induction of p53 in p53-positive A549 cells. As shown in Fig. 6A, western blot analysis established that a 36 h exposure to CAV caused p53 protein induction in a dose-dependent manner. In the time course study (Fig. 6B), p53 was induced as early as 6 h after the addition of CAV. Induction of p53 protein was prior to the induction of p21/WAF1 mRNA at 12 h after CAV treatment (Fig. 5B). The p21/WAF1 mRNA was not induced by 6 h treatment with CAV (data not shown). These results suggested that induction of p53 by CAV could act as a transcriptional activator of the *p21/WAF1* gene and mediate the induction of *p21/WAF1* in A549 cells.

DISCUSSION

Our investigation has established that CAV could inhibit the proliferation of A549 human lung adenocarcinoma cells by arresting the cell cycle at G1 phase. Although a previous report indicated that CAV could be responsible for increasing the amount of p53 protein in human fibroblasts,[31] we have established that the G1 phase arrest is due to the induction of the CDK inhibitor, p21/WAF1. The prior induction of p53 could, at least in part, explain the p21/WAF1 induction by CAV. Subsequently, the *RB* gene product was dephosphorylated, and this might have resulted in G1 phase arrest.

We also observed that CAV inhibits the cellular proliferation of HeLa or HTB9 cells, which of both lack intact *p53* gene. We found no induction of *p21/WAF1* gene or G1 phase arrest by CAV in these two cell lines.

These results are consistent with our hypothesis that induction of p21/WAF1 and G1 arrest by CAV is p53-dependent. However, these results also show that CAV can suppress cellular proliferation through a p53-p21/WAF1-independent pathway. As another possibility, CAV could directly or indirectly dephosphorylate RB protein to cause G1 arrest. More study will be required to clarify these questions.

CAV is well known as an inducible nitric oxide synthase (NOS) inhibitor,[12, 13] suggesting that the treatment of A549 cells with CAV might decrease their production of NO. It is relevant to consider whether the induction of p53 was due to a direct effect of the NOS inhibitor or to an attenuation of the NO level. We investigated if other known NOS inhibitors such as aminoguanidine and NG-nitro-L-arginine could inhibit cell proliferation. In contrast with the growth-inhibitory effect of CAV on A549 cells, these aforementioned NOS inhibitors did not inhibit the growth of A549 cells at a concentration of 2 to 3 m*M* or more (data not shown). These results suggested that a decrease in NO production did not cause A549 cell growth inhibition. Furthermore, other workers have reported that NO itself caused an accumulation of p53.[32, 33] Therefore, the p53 induction by CAV demonstrated by our study could not have been due to an inhibitory effect of CAV on NOS. Further study will be required to clarify the mechanism of p53 enhancement by CAV.

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