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L-arginine enhances arginine deiminase induced human lymphoma cell growth inhibition through NF-κBp65 and p53 expression *in vitro*

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Abstract. OBJECTIVE: Arginine deiminase (ADI) and L-arginine (L-Arg) can act as anti-tumor agents *in-vitro* and *in-vivo*. However, the mechanism of ADI and L-Arg as anti-tumor agents has not been clearly shown.

MATERIALS AND METHODS: With the goal of understanding the role of ADI and L-Arg in inhibition of cell growth, we used the Ramos human lymphoma cell line, which is known to be ADI-sensitive, and observed the p53 and NF-κBp65 protein expression after ADI and arginine treatment. After determining an optimal experimental ADI concentration (0.01 U/ml), we studied the effects of ADI treatment, when combined with different concentrations of L-arginine (control, ADI only, ADI with 10 μM/ml Arg, ADI with 30 μM/ml Arg, and ADI with 50 μM/ml Arg). An MTT assay was used to assess cell survival after treatment, Western blot analysis to determine the levels of the NF-κBp65, p53 and NO mediators and nitric oxide assays were used to determine nitrite levels.

RESULTS AND CONCLUSIONS: L-arginine enhanced ADI-induced inhibited cell growth through expression of NF-κBp65 and p53 in a dose-dependent manner.

Keywords:
L-Arginine, Arginine deiminase, Human lymphoma cell, NF- Bp65, p53.

Introduction

Arginine deiminase (ADI) is an enzyme degrading arginine and citrulline generated catalytic arginine. ADI is used as a drug treatment for arginine-deficient tumors (e.g., carcinoma, melanoma). ADI is an enzyme that catalyzes the hydrolysis of arginine into citrulline and ammonia[1,2], thereby, cutting off supply of this nutrient to tumor cells, inhibiting arginine-deficient tumor cell proliferation, but has no effect on normal cells[3]. ADI causes arginine depletion enzymatically and, thus, ADI can block inducibile nitric oxide sinthase-(iNOS)-induced NO production while increasing endothelial nitric oxide synthase-(eNOS)-mediated NO production[4,5]. Moreover, ADI derived from Mycoplasma has been reported to induce apoptosis in a variety of human cancer cells *in-vitro*[6], and to exert a potent anti-tumor activity *in vivo*[7]. The expression of NOS in colon carcinoma cells stimulates the growth of cells carrying a mutant p53 such as HT-29, whereas in cells with wild-type p53, there is an inverse association between NOS activity, cell growth and the likelihood of tumor development[8,9]. It has been postulated[10] that the anti-tumor action of ADI is mediated by the direct inhibition of tumor cell growth via the down-regulation of polyamine-biosynthesis in tumor cells. Also other study[11] suggests that the inhibition of *denovo* protein synthesis is the likely mechanism for the inhibitory effect of ADI on the proliferation of ADI-sensitive tumor cells.

L-Arginine (L-Arg) is a non-essential amino acid and an important mammal nutrient, which is derived from the urea cycle, and is also as a major non-glycolytic energy source in various microorganisms[12,13]. Arginine significantly inhibits the growth of most tumors and leads to apoptosis[14], it is also the precursor of nitric oxide (NO) generated within cells. NO is synthesized from arginine by the catalytic action of a group of enzymes termed the nitric oxide synthases (NOS). NO is a second messenger molecule that is endogenously produced by the family of NO synthases, and has been shown to be involved in a variety of biological signaling processes. NO plays an important role in tumor growth by regulating endothelial cell growth factor, fibroblast growth factor, and matrix metalloproteinase activity[15,16]. Moreover, NO is able to act in a dual manner, leading either to an induction of apoptosis or to the blunted execution of apoptosis in cancer cells.

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We set out to examine the hypothesis that ADI-induced inhibition of cell growth might be affected by an NO donor, such as arginine. We selected the ADI-sensitive Ramos cell line, which is a human lymphoma cell line, for this study and examined the role of NF-κB p65 and p53, to discover if NO acts as a mediator to induce apoptosis, and arginine enhanced ADI cell growth inhibition, which are associated with NF-κB p65 and p53.

Materials and Methods

Cell and Cell Culture

Ramos human lymphoma cells (ATCC CRL-1596, SIBS, CAS) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% glutamine, 100 u/ml penicillin/streptomycin (Gibco-BRL,Grand Island, NY, USA), at 37°C, in a 5% CO_2 humidified incubator.

Recombinant ADI Purification

The *Escherichia coli* strain NovaBlue (DE3), which was transformed with the plasmid pET32-ADI, and cultured at 37°C in 500 ml of LB media containing 50 mg/ml ampicillin (Boehringer Mannheim, Germany) to an absorbance of 0.4 at 600 nm. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1.0 mM. The cells were harvested by centrifugation at 6,000×g for 20 min at 4°C after a 4h induction by IPTG at 32°C. The bacterial pellet was then resuspended in 100 ml of 10 mM potassium phosphate buffer (pH7.4) containing 1 mM EDTA, and then disrupted by sonication. The resulting lysate containing rADI was collected by centrifugation at 12,000×g for 20 min at 4°C, and washed twice with a buffer (10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, and 4% Triton X-100). Inclusion bodies that contained rADI were solubilized in a denaturing buffer (30 mM Tris-HCl, pH 8.5, 6 M guanidine-HCl, and 10 mM dithiothreitol) for 1h at 37°C, as described elsewhere. The solubilized proteins were refolded in a tenfold excess volume of 10 mM potassium phosphate buffer at 15°C for 24h after removing cell debris by centrifugation. Purification of ADI from the renaturation mixture was performed using DEAE and phenyl-sepharose, and this was followed by arginine-affinity column chromatography, as described previously[17]. The purified rADI was analyzed by SDS-PAGE (12.5%) and by gradient gel electrophoresis (5-20%). Table I provides a summary of the purification of ADI expressed by *E. coli*.

Nitric Oxide Assay

The concentrations of nitrites in Ramos human lymphoma cell supernatants were determined by measurement of NO production, and were determined in individual, cell-free samples incubated for 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). After incubation for 10 min at room temperature, the optical density of the samples was measured using a Molecular Device microplate reader at 570 nm.

ADI and Arginine Treatment

Ramos human lymphoma cells were seeded 1×10^5 into 10 cm culture dishes. Three days later they were treated with ADI at 0.1, 0.05, 0.01, 0.005 or 0.001 U/ml. After 24 and 48h, we measured inhibition of cell growth by simply counting the non-adherent Ramos cells. The ADI concentration was fixed at 0.01 U/ml for the next experimental step. Arginine (Sigma, St. Louis, MO) was used as the NO donor. We classified the treated cell into five groups according to their ADI and arginine treatment, as follows: untreated control, ADI (0.01 U/ml) treatment without Arg, ADI with 10 μM/ml Arg, ADI with 30 μM/ml Arg, or ADI with 50 μM/ml Arg. Cells were treated with the respective ADI and Arg concentrations for 24 h and each experiment was repeated five times.

Table I. Summary of purification of ADI expressed in *E. coli*.

Preparation	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)
Inclusion body	312.5			
Refolding	57.2	438.2	12.4	100.0
DEAE-Sepharose	7.8	357.6	58.7	87.5
Arginine-Sepharose	6.1	327.5	76.3	82.6

Treatment with NK-κB p65 siRNA and p53 siRNA

siRNA to NK-κB p65 was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), p53 siRNA (targeting 5'-TTCTCTCCC-CAACAATGAGG3' specific for p53) and double strand control RNA was synthesized by Invitrogen (Carlsbad, CA, USA). Ramos human lymphoma cells were transfected with 50 nM of control siRNA, 50 nM of NK-κB p65 siRNA, 50 nM of p53 siRNA, or 50 nM of NK-κB p65 siRNA plus 50 nM of p53 siRNA using Lipofectamine 1000 reagent. 24 hours after transfection, cells were changed to medium supplemented with ADI (0.01 U/ml) alone or a combination of ADI and an increasing concentration of Arg (10, 30, 50 μM/ml), and after 24 hours the number of viable cells was determined in triplicate wells using a trypan blue exclusion assay.

Western Blot Assay

Cells were harvested by centrifugation and then lysed in RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton-X, and 100 mM phenylmethylsulfonyl fluoride 4 μl/ml. After centrifugation at 14,000 rpm for 30 min, protein concentrations in cell lysates were determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and cell lysates were then stored at –80°C. Proteins were electrophoresed on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and then detected by anti-p53 antibody (sc-7195, Santa Cruz, CA, USA) and anti-NFκB p65 antibody (sc-372, Santa Cruz, CA, USA) with enhanced chemiluminescence (Amersham Pharmacia Biotech, UK). Rabbit anti-β-actin antibody (H-196, Santa Cruz, CA, USA) was used as an internal control.

Statistical Analysis

A detailed statistical analysis was performed using Statistica for Windows 10.0. The results were expressed as arithmetic means and standard errors. Continuous variables were assessed for normal distribution with the use of the Shapiro-Wilk test. Differences between means were analyzed by oneway analysis of variance, followed by a post hoc Tukey's test. Avalue of $p < 0.05$ was regarded as representing a significant difference.

Results

The Ratio of Surviving Cell Treatment with ADI

The Ramos cells were treated with various concentrations of ADI (control, 0.001, 0.005, 0.01, 0.05 and 0.1 U/ml) for 24 and 48 h, and an ADI concentration of 0.001 U/ml was chosen for the 48h treatment schedule. The surviving cell fraction was 62% after 24h treatment with 0.01 U/ml ADI and 15% after 48h treatment (Figure 1).

Cell Growth Inhibition Treatment with ADI and L-arginine

The cell count of Ramos cells treated with 0.01 U/ml ADI was reduced to 24.7% of the control group cell count after 48h of treatment. However, when different concentrations of L-Arg were added (10, 30, and 50 μM/ml), cell count was reduced in a dose-dependent manner (Figure 2). Although full reduced was not observed, the reduction resulted of 10, 30 and 50 μM/ml L-Arg in 26.5%, 37.3 % and 52.6% respectively.

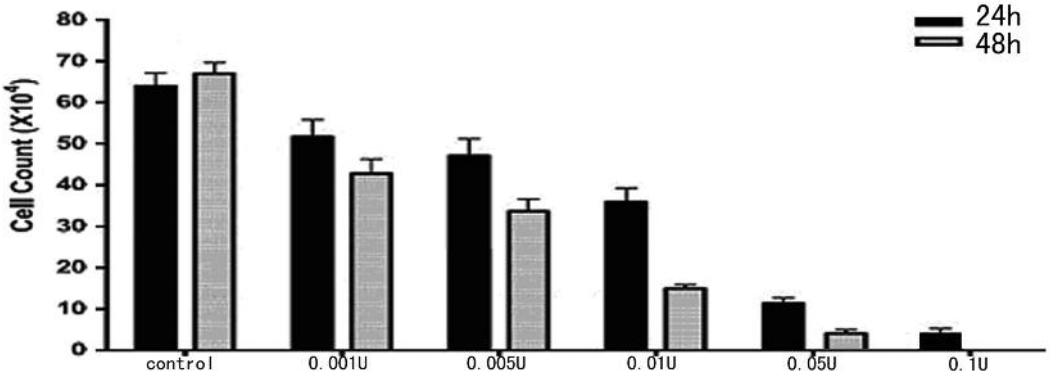

Figure 1. Treatment with Various ADI concentrations affect Ramos cells counts. Cell counts of ADI-treated Ramos cells according to various ADI concentrations (ADI 0.1, 0.05, 0.01, 0.005 or 0.001U/ml) and treatment durations (24 and 48h).

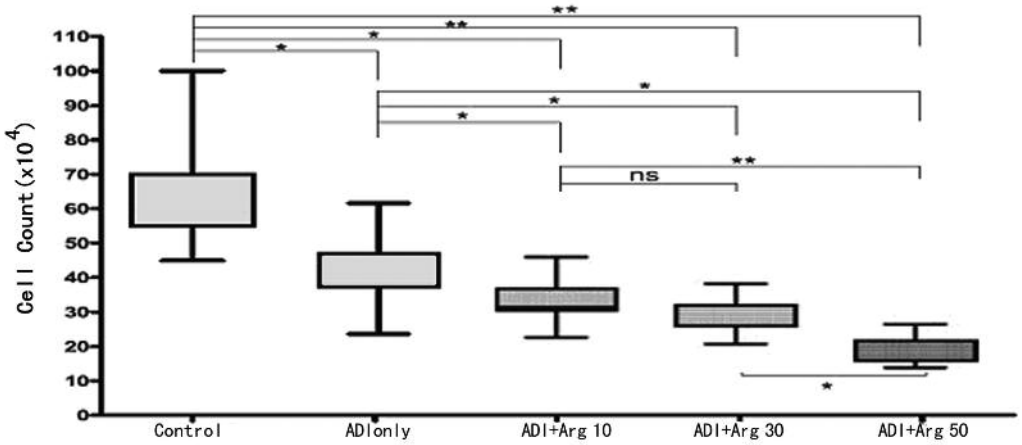

Figure 2. Extrinsic L-arginine enhance ADI-induced cell growth inhibition in a dose dependent pattern. Extrinsic L-arginine enhance ADI-induced cell growth inhibition in a dose dependent pattern. Cell counts were taken after 24 h. ADI only: ADI 0.01 U/ml only, ADI+ Arg 10: ADI 0.01 U/ml + Arg10 μM/ml, ADI+ Arg30: ADI 0.01 U/ml + Arg 30 μM/ml, ADI+ Arg50: ADI 0.01 U/ml + Arg 50 μM/ml. ***p* value < 0.001, **p* value < 0.05, ns: *p* value > 0.05.

Extrinsic L-arginine Increased Nitrite Concentrations

Nitrite concentrations between the control group and the ADI-only treatment group were not significantly different. In addition, citrulline treatment did not affect nitrite concentration (data not shown). As we expected, nitrite concentrations increased with extrinsic L-arginine treatment (10, 30 and 50 μM/ml) in a dose-dependent manner (Figure 3).

Western Blot Analysis of p53 and NF-κB p65

p53 expression was markedly reduced in the ADI only treatment group and p53 expression was markedly increased treatment with L-Arg. Conversely, NF-κBp65 expression was markedly increased in the ADI-only treatment group and NF-κBp65 expression was markedly reduced treatment with L-Arg (Figure 4).

Western Blot Analysis of NF-κB p65 and/or p53

To determine whether NF-κBp65 or p53 are directly involved in ADI-mediated apoptosis, we treated each of the experimental groups with NF-κB p65 siRNA and/or p53siRNA. Interestingly, when we treated cells only with p53siRNA, ADI-mediated apoptosis was markedly increased, however, ADI-mediated apoptosis was markedly decreased when we treated cells only

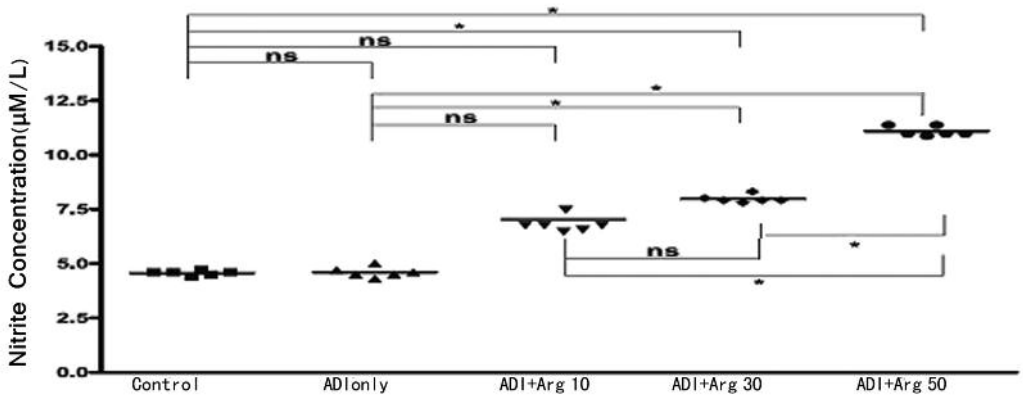

Figure 3. Nitrite concentration change after treatment with various concentrations of extrinsic L-arginine. Nitrite concentration change after treatment with various concentrations of extrinsic L-arginine. The nitric oxide assay was performed after 24 h. ADI only: ADI 0.01 U/ml only, ADI+ Arg10: ADI 0.01 U/ml + Arg 10 μM/ml, ADI+ Arg 30: ADI 0.01 U/ml + Arg 30 μM/ml, ADI+ Arg 50: ADI 0.01 U/ml + Arg 50 μM/ml. **p* value < 0.05, ns: *p* value > 0.05.

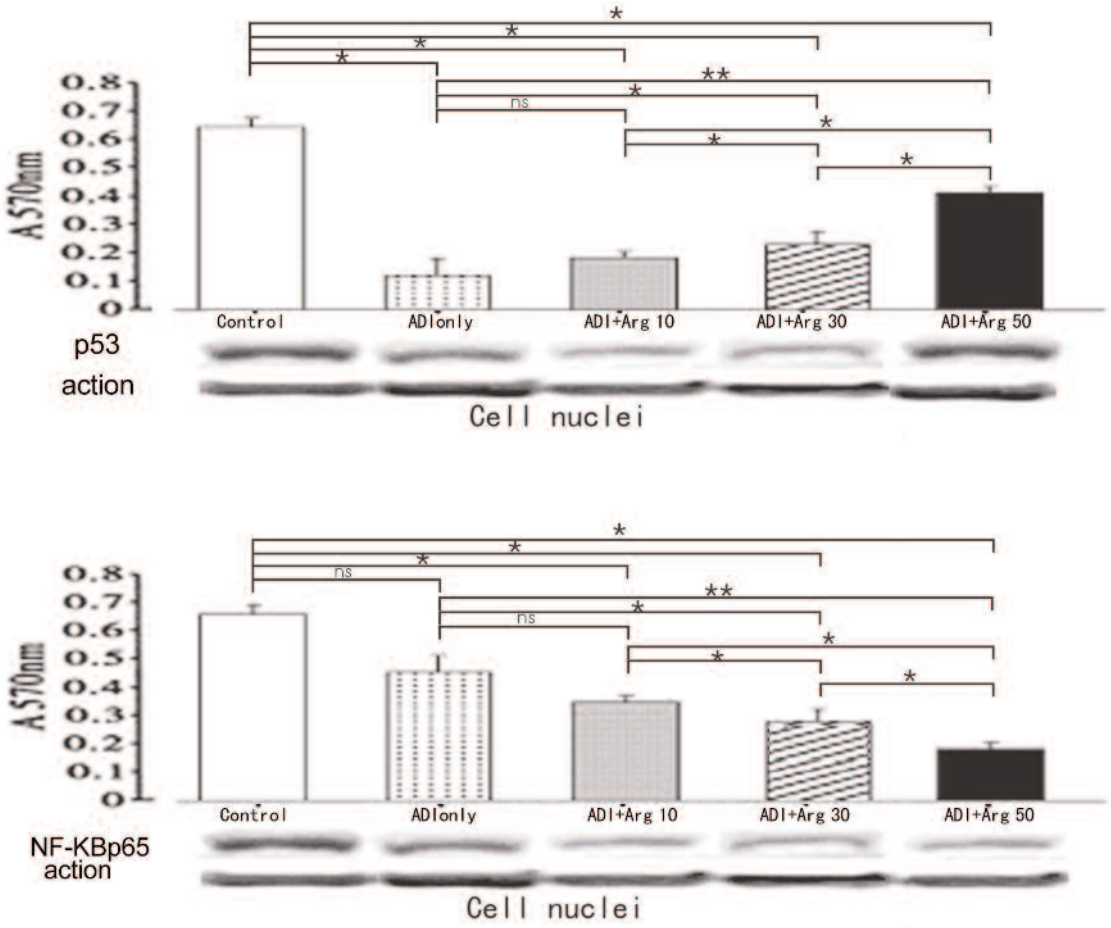

Figure 4. The expression of p53 and NF-κBp65 was affected with ADI only treatment and with L-Arg. Western blot analysis of p53 and NF-κBp65. 1 Control, 2 ADI 0.01 U/ml only, 3 ADI 0.01 U/ml + Arg 10 μM/ml, 4 ADI 0.01 U/ml + Arg 30 μM/ml, 5 ADI 0.01 U/ml + Arg 50 μM/ml. **p value < 0.001, *p value < 0.05, ns: p value > 0.05.

with NF-κBp65siRNA. Furthermore, when we simultaneously treated cells with p53siRNA and NF-κB p65siRNA, ADI mediated apoptosis was furtherly increased (Figures 5, 6).

Discussion

ADI can inhibit cell proliferation through several different mechanisms, such as by arresting the cell cycle[18], by having an effect on antiangiogenesis and induced cells apoptosis through depletion of arginine. NO is synthesized from L-arginine by the catalytic action of a group of enzymes termed nitric oxide synthases (NOS). There are three known NOS isozymes, such as the neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms are constitutively expressed. However, the iNOS isoform is non-constitutively expressed[19,20]. NO may play a role in the survival and progression of lymphoid tumor[17]. A study has reported that NO is produced independently of prolactin (PRL) and promotes Nb2 lymphoma cell line survival[21]. Furthermore, NO could effectively inhibits apoptosis through scavenging superoxide anions generated in the mitochondria of p53 mutant cells[22,23], thereby, preventing the down-regulation of anti-apoptotic factors NF-κBp65 and Bcl-XL, which control the mitochondrial apoptotic pathway[24]. We hypothesize that lymphoid tumors may be sensitive to ADI treatment, and that ADI-induced the expression p53 and NFκBp65; this action was enhanced by addition of extrinsic arginine.

In the present study, we found that ADI suppresses cell growth in the Ramos lymphoma cell line in a dose-dependent manner. However, it had not been previously reported that L-arginine can affect ADI-inhibition of cell growth. ADI can cat-

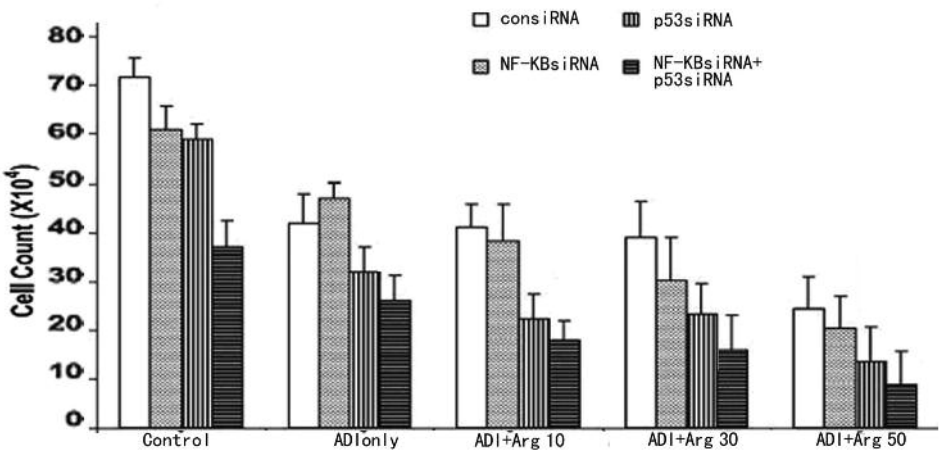

Figure 5. To determine whether NF-κB p65 and/or p53 are directly involved in ADI-mediated apoptosis, we used NF-κBp65 siRNA and/or p53 siRNA. Cell counts were taken after 48 h. ADI only: ADI 0.01 U/ml only, ADI+ Arg 10: ADI 0.01 U/ml + Arg 10 μM/ml, ADI+ Arg 30: ADI 0.01 U/ml + Arg 30 μM/ml, ADI+ Arg 50: ADI 0.01 U/ml + Arg 50 μM/ml.

alyze the hydrolysis of extracellular L-arginine into L-citrulline and ammonia through the arginine dihydrolase pathway. However, we could not find any changes in the nitrite concentration in the ADI only treatment group. Therefore, we suggest that the mechanism of ADI-induced cell growth inhibition is not mediated by deprivation decrease in NO production. However, when we treated with both ADI and L-arginine, cell growth inhibition gradually increased, at the same time, the

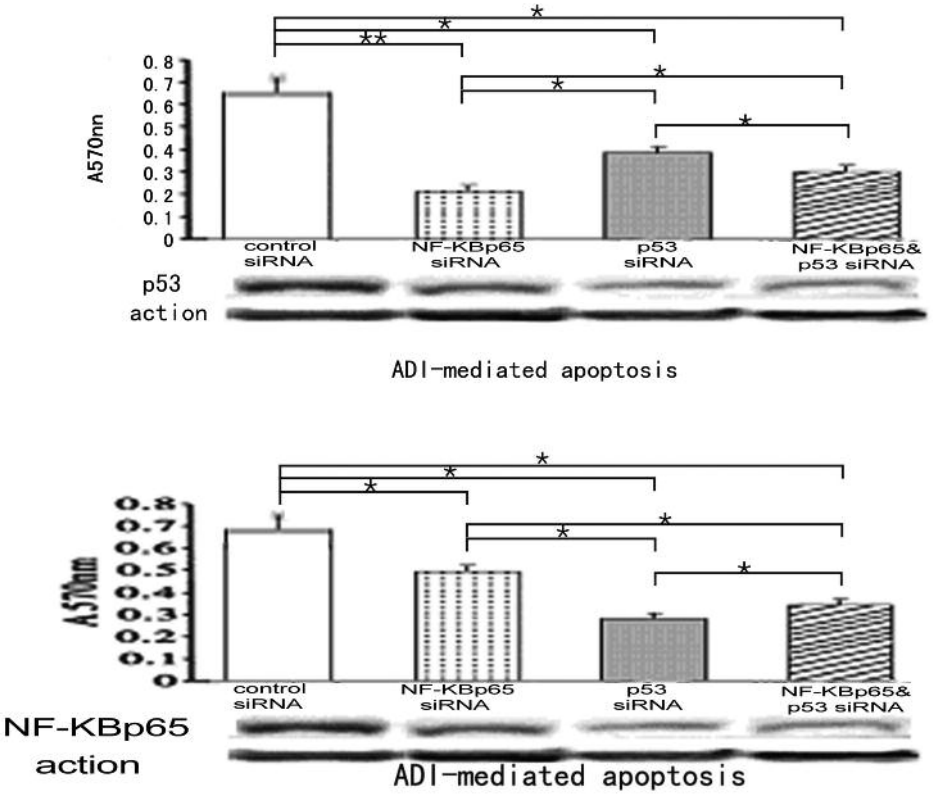

Figure 6. Western blot analysis of NF-κB p65 and p53 for each treatment group (control siRNA, p53 siRNA, NF-κB p65siRNA, p53 siRNA and NF-κB p65). $^{**}p$ value < 0.001, $^{*}p$ value < 0.05, ns: p value > 0.05.

protein expression levels of NF-κBp65 were down-regulated, and at the same time the protein expression levels of p53 increased in a L-arginine dose-dependent manner. Our preliminary data suggest that arginine inhibits cancer cell growth, and induces apoptosis through the raised expression of p53 protein and regulation of intracellular constitutive activation of NF-κBp65.

We examined the protein expression levels of NF-κBp65 and p53 which are two mediators of NO, known to be upregulated in apoptosis. When we treated cells with ADI only, we observed a marked decrease in NF-κBp65 and a significant increase in p53 expression. Therefore, our data suggest that ADI-induced inhibition of cell growth is mediated by expression of NO mediators, such as NF-κBp65 and p53. But we also observed a significant increase in p53 and a significant decrease in NF-κBp65 expression after extrinsic L-arginine treatment, over cells-treated with only ADI. Furthermore, treatment with p53 siRNA resulted in a marked increase in ADI-mediated apoptosis, while simultaneous treatment with p53 siRNA and NF-κBp65siRNA gradually increased ADI-mediated apoptosis. Therefore, we suggest that NF-κBp65 and/or p53 directly involved in ADI-mediated apoptosis.

Conclusions

ADI inhibits cell proliferation through several mechanisms, such as raised the expression of protein p53 and down-regulated the expression of protein NF-κBp65 during arginine deiminase treatment of Ramos cell. Furthermore, arginine can enhance ADI-induced inhibition of cell growth.

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Conflict of interest

The authors declare that they have no conflict of interest

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