Anti-tumor activity of arginine deiminase *via* arginine deprivation in retinoblastoma

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Abstract. In spite of recent advances in the treatment of retinoblastoma, chemotherapy is still challenging in high-stage intraocular retinoblastoma or metastatic retinoblastoma. Here, we investigated whether arginine deprivation *via* arginine deiminase (ADI) could be a new anti-tumor therapy in retinoblastoma cells. Expression of argininosuccinate synthetase (ASS) was detected in human retinoblastoma tissues. Even with a high expression of ASS, ADI effectively inhibited the proliferation of retinoblastoma cells and induced retinoblastoma cell death in a dose-dependent manner. These results indicate that arginine deprivation *via* ADI could be another treatment option for retinoblastoma due to low ASS activity in retinoblastoma cells.

Introduction

Retinoblastoma is the most common intraocular cancer in children (1). Both hereditary and non-hereditary retinoblastomas result from the inactivation of the representative tumor suppressor gene, *RB1* (2). Treatment options including enucleation, radiation, chemotherapy and additional local treatments of cryotherapy, laser photocoagulation, and thermotherapy have been successful in saving lives. Usually, patients with unilateral retinoblastoma undergo enucleation, whereas children with bilateral retinoblastoma often undergo anti-cancer therapy to avoid bilateral enucleation and blindness. Recently, chemotherapy combined with local treatments has improved the rate of eye salvage or vision preservation (3-5). A currently used chemotherapy regimen for retinoblastoma is based on carboplatin and etoposide, which originated from another rare childhood solid cancer of the central nervous system (6). However, chemotherapy is still challenging in high-stage intraocular retinoblastoma or metastatic retinoblastoma. For years, alternative chemotherapeutic combinations have not been identified. Moreover, etoposide has been known to increase acute myeloblastic leukemia as a secondary malignancy which is likely due to its topoisomerase II inhibitory effect (7).

Amino acid deprivation therapy has been encouraged in the field of cancer treatment. Amino acid degrading enzymes, such as paraginase and methioninase, have been encouraged as potent anti-tumor agents (8,9). Similarly, arginine is a semi-essential amino acid to be depleted in normal cell metabolism (10) and arginine deiminase (ADI) is an arginine degrading enzyme that catalyzes the hydrolysis of arginine into citrulline and ammonia. We and other research groups showed that arginine deprivation by ADI could be a possible cancer treatment (11-16). Arginine-auxotrophic tumor cells cannot survive in an arginine-deprived condition, but in fact, some cells can live well with ADI, because citrulline could be converted to arginine in 2 steps by using enzymes arginino-succinate synthetase (ASS) and argininosuccinate lyase (ASL) (17). ASS is known as the rate-limiting enzyme in the conversion of citrulline into arginine, so anti-tumor activity of ADI generally depends on the cellular ASS activity and citrulline to arginine recycling (18).

In this study, we investigated whether ADI has anti-tumor activity in retinoblastoma cells. To examine this hypothesis further, we checked the expression of ASS in human retinoblastoma tissues and retinoblastoma cell lines. To evaluate both the anti-tumor activity of ADI and ASS activity in retinoblastoma cells, we compared cell growth and lactate dehydrogenase (LDH) release in ADI-treated cells to that in L-arg deprived cells without ADI treatment.

Materials and methods

Materials. Recombinant arginine deiminase (rADI) was purified in our laboratory as previously described (15). Briefly, rADI was purified to homogeneity on SDS-PAGE from inclusion bodies accumulated in the cytoplasm of *E. coli* BL21

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(DE3) (Novagen, Madison, WI) transformed with pET32a-ADI and had a specificity of 50 units/mg protein. One unit of ADI is the amount of enzyme catalyzing 1 μmole of arginine to citrulline per min at 37˚C under the assay conditions.

Tissue preparation and immunohistochemistry. All human retinoblastoma tissue samples were obtained with informed consent, institutional review board approval, and in accordance with the tenets of the Declaration of Helsinki. Primarily enucleated eyeballs from patients diagnosed with retinoblastoma were obtained from the Seoul National University Children's Hospital. After nucleation, the eyeballs were fixed by immersion in Carnoy's solution for 2 h at room temperature. Each specimen was then dehydrated through a series of graded ethanol solutions and embedded in paraffin using standard techniques. Paraffin-embedded eyes were sectioned into 4 μm sections, and sections were then mounted on slides coated with 0.5% Elmer's glue for immunohistochemistry. Immunohistochemistry was performed using an Inno-Genex (San Ramon, CA) immunohistochemistry kit. For ASS immunostaining, anti-ASS monoclonal antibody (BD Biosciences, Bedford, MA) was used at the concentration of 1:200 and TRITC-conjugated IgG (1:400, Jackson ImmunoResearch) were used as secondary antibodies.

Cell culture. Human mammary adenocarcinoma (MCF-7) cells were generously donated by Dr Min and the Y79 retinoblastoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Retinoblastoma SNUOT-Rb1 cells were established from a 3-year old retinoblastoma patient, who was diagnosed with a primary retinoblastoma at Seoul National University Children's Hospital (Korea). MCF-7 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Gibco BRL, Rockville, MD), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco BRL). Retinoblastoma cells were cultured in RPMI-1640 (Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37˚C. For LDH assay, L-arg-free DMEM medium was a product of Gibco BRL and L-arg-free/L-cit medium was supplemented with 1 mM L-citrullin and 1 mM ammonium chloride (Sigma). For experiments in this report, the L-arg-free DMEM medium is designated as 'L-arg-free DMEM medium', and L-arg-free DMEM medium supplemented with 1 mM L-arg is designated as a 'control medium'.

Western blot analysis. Western blotting was performed using the standard Western blotting methods. The protein concentration in the cytosolic fraction was measured using a BCA protein assay kit (Pierce, Rockford, IL). For Western blot analysis, anti-ASS monoclonal antibody was used at a concentration of 1:1000 (BD Biosciences), and horseradish peroxidase-conjugated anti-mouse IgG was used at 1:10,000 dilution. To ensure the equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against ß-actin.

Reverse transcription-polymerase chain reaction. Total RNA was prepared from cells using TRIzol reagent (Invitrogen, New Zealand), according to the manufacturer's protocol. RNA was dissolved in diethyl pytocarbonate (DEPC)-H_2O and first-strand cDNA synthesis was performed using Super Script™ II Rnase H-reverse transcriptase (Invitrogen) and an oligo-dT primer. The cDNA was subjected to 30 cycles of amplification using the primers; ASS Forward 5'- CCTGAT GGAGTACGCAAAGC-3' and Reverse 5'- ACACCAGCTC AGCAAATTTC-3', ASL Forward 5'-GCTCCGAGCAGAA CTCAACT-3' and Reverse 5'-TTTGCGGACCAGGTAAT AGG-3', GAPDH Forward 5'-AAGGTCATCCCTGAGCT GAA-3' and Reverse 5'-CCCCTCTTCAAGGGGTCTAC-3' at 95˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min. The PCR products were analyzed on 1% agarose gels stained with ethidium bromide.

Cell proliferation assay. Cells ($1x10^5$) were seeded in 12-well culture plates, and then treated with various concentrations of ADI (0.25-5 mU/ml). After 3 days, aliquots of cells were mixed with trypan blue dye (1:1) and loaded onto a haemocytometer. Total cell number was counted and dead cells were specifically identified as those that had taken up the blue dye. The percentage of dead cells was then calculated as a proportion of the total cell number.

Cell cytotoxicity. This was assessed using the LDH assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Cells ($1x10^4$) were seeded in a 96-well plate (Nunc, Roskilde, Denmark) in 10% FBS DMEM medium or L-arg-free medium supplement with L-citrullin and ammonium chloride. After treatment with various concentrations of ADI (0.25-5 mU/ml) for 3 days, the culture medium was mixed with an equal volume of LDH substrate solution in the dark for 30 min. The reaction was stopped with 1 M acetic acid, and the absorbance was measured at 490 nm.

Statistical analysis. Data are expressed as mean ± S.D. Comparisons between the controls and treated groups were performed using the Student's t-test. $P<0.05$ was considered statistically significant.

Results

Expression of ASS in human retinoblastoma tissues. ASS was immunoreactive in all retinoblastoma tissues of 20 patients, which were randomly selected and examined (Fig. 1). ASS-positive viable cells were widely distributed over the tumor, but were not co-localized with DAPI-positive cells even though these existed around the rosettes.

ASS and ASL are expressed in retinoblastoma cell lines. To evaluate eligibility for arginine deprivation therapy with ADI, the expression levels of ASS was measured in retinoblastoma cell lines by Western blot analysis. Previously, we showed that ASS expression is relatively high in MCF-7 cells (16), and thus we used its levels as a positive control. We found that ASS is expressed at a high level in retinoblastoma cell lines, such as Y79 and SNUOT-Rb1, comparable to the levels seen in MCF-7 (Fig. 2A). We also confirmed ASL expression in retinoblastoma cell lines by RT-PCR (Fig. 2B). The expression level of ASS was high in retinoblastoma cell lines, which

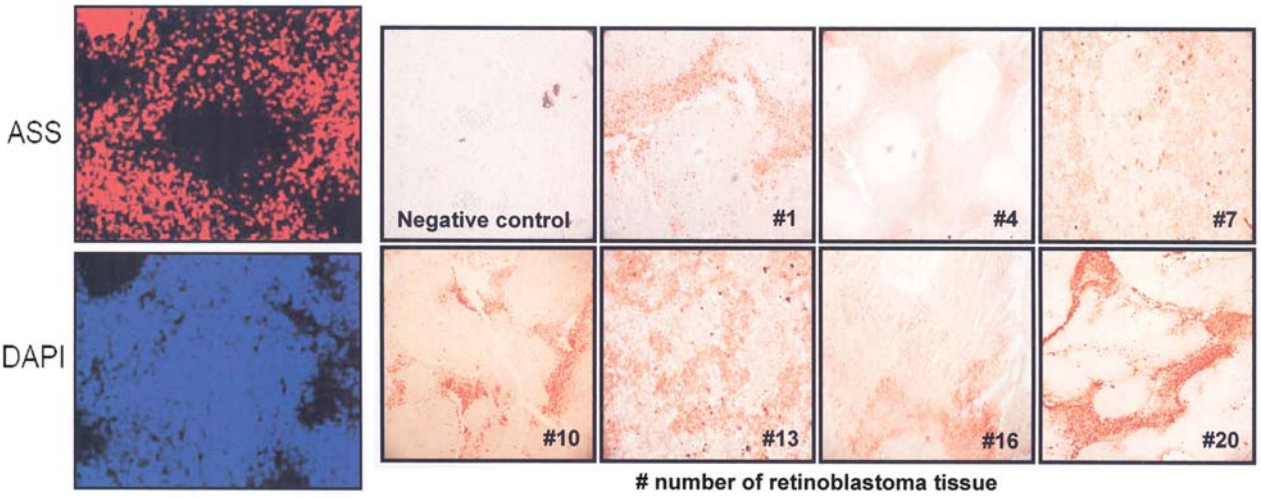

Figure 1. Immunohistochemistry of ASS in human retinoblastoma tumors. Tumor tissues from 20 patients were subjected to immunohistochemistry and analyzed for ASS expression. #, the number of retinoblastoma tissue.

Figure 2. Expression of ASS and ASL in retinoblastoma cell lines (Y79, SNUOT-Rb1) and MCF-7 cells. (A) Protein samples of each cell were resolved on 12% SDS-PAGE and Western blot analysis was performed using a human anti-ASS monoclonal antibody. MCF-7 cells were used as a positive control. (B) Total RNA was extracted from the cells and then first-strand cDNA was synthesized from 1 μg of total RNA by using reverse transcriptase and oligo-dT. Amplification of mRNA of ASS and ASL was carried out as described in Materials and methods.

suggests that arginine deprivation therapy *via* ADI may not be applicable to retinoblastoma.

ADI inhibits proliferation and induces cell death of retinoblastoma cells. We treated retinoblatoma cells (SNUOT-Rb1, Y79) with ADI (0.25-5 mU/ml) for 72 h and measured cell growth by the trypan blue exclusion method (Fig. 3A). A significant suppression in cell proliferation was observed in both retinoblastoma cell lines treated with ADI in a dose-dependent manner. To test the possibility that ADI treatment may cause cell death, LDH released in culture media was measured. Fig. 3B shows that levels of LDH release is increased by ADI treatment in SNUOT-Rb1 and Y79 cells, while there are no changes in MCF-7 cells.

ASS activity is lacking in retinoblastoma cells. Although ASS expression is relatively high in retinoblastoma cells, ADI-induced arginine deprivation inhibits proliferation and induces cell death (Fig. 3). This could be due to the lack of ASS activity in retinoblastoma cells. To evaluate both anti-tumor activity of ADI and ASS activity in retinoblastoma cells, we examined LDH release i) in the control medium with or without ADI, and ii) in the control medium with ADI or L-arg-free medium without ADI. With the treatment of ADI, LDH release was significantly increased in retinoblastoma cells, especially in SNUOT-Rb1 cells, but slightly increased in MCF-7 cells (Fig. 4), where ASS is highly expressed similarly to retinoblastoma cells (Fig. 2A). The amount of LDH release by arginine deprivation from culture media was similar to that of ADI-induced arginine deprivation. These data suggest that ADI-induced retinoblastoma cell death could result from arginine deprivation, as retinoblastoma cells lack ASS activity even at high expression of ASS.

Discussion

Retinoblastoma is the most common malignant tumor of the retina and usually occurs in children below 4 years of age. It is an aggressive tumor that can lead to loss of vision and the eye, and in extreme cases, to death. Treatment methods depend on the stage of the disease, which can include surgical enucleation of the afflicted eye, local therapy (episcleral brachytherapy, cryotherapy, laser photocoagulation, or transpupillary thermotherapy), chemotherapy and/or external beam radiotherapy. To avoid or delay radiation therapy, the use of chemotherapy in conjunction with local therapy for tumor reduction has been investigated. Several agents, including vincristine, cyclophosphamide, doxorubicin, epipodophyllotoxins, and platinum-based products, have proven effective in metastatic retinoblastoma (3,19-24), however, with the increasing incidence of the systemic toxicity, in particular, epipodophyllotoxin-related leukemias (25), new chemotherapy protocols should be developed.

In the present study, we investigated whether the arginine deprivation by ADI treatment could be another treatment option for retinoblastoma. Amino acid deprivation therapy has been encouraged in the field of cancer treatment, because amino acid deprivation cannot introduce toxins, but can induce physiological and metabolic imbalances (26). Of the 20 or so amino acids that could be manipulated, arginine was the first to be seriously explored, but methionine, tryptophan, phenylalanine, and other amino acids have had their champions (27). Because, arginine is a semi-essential amino

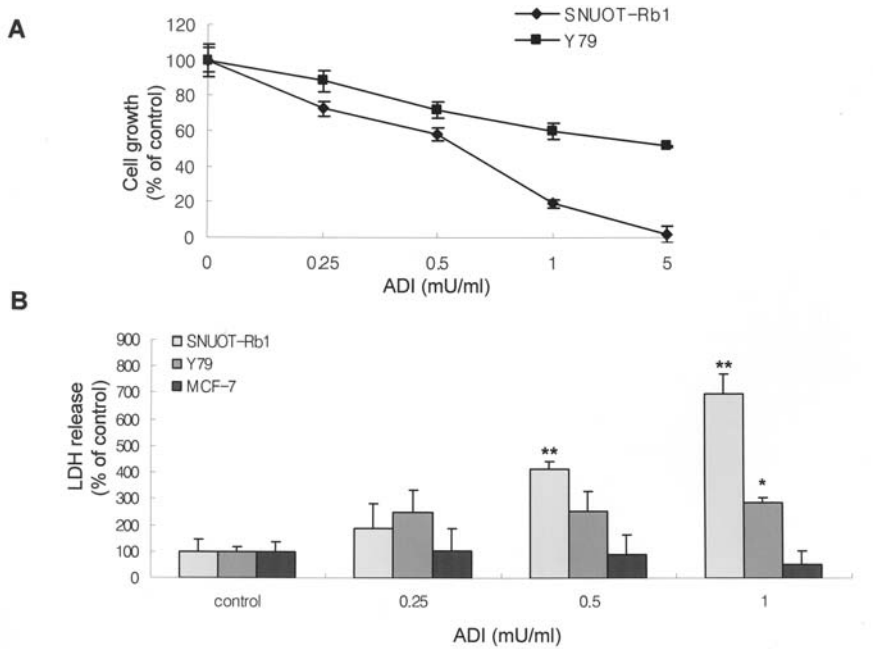

Figure 3. Effect of ADI on the growth and viability of retinoblastoma cells. Cells were seeded in arginine containing medium and then treated with increasing concentrations of ADI (0.25-5 mU/ml) for 72 h. (A) Cells were stained with trypan blue and counted with a haemocytometer. (B) LDH release in cell culture media was measured. The data shown are the means of triplicate cultures of each cell and are presented as a percentage of control cells that received medium only. *P<0.005. **P<0.0005.

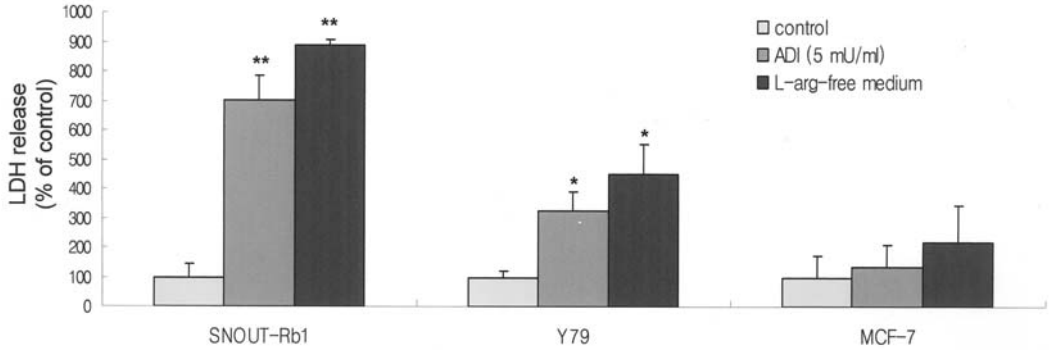

Figure 4. The effect of arginine-free media on retinoblastoma cells. Cells were cultured in complete medium (as 100%), 5 mU/ml ADI containing medium, or L-arg-free medium. After a 3-day incubation, LDH release in cell culture media was measured. The data shown are the means of triplicate cultures of each cell type and are presented as a percentage of control cells that received medium only. *P<0.005. **P<0.0005.

acid to be depleted in normal cell metabolism (10) and is the substrate of numerous enzymes, such as arginase, arginine decarboxylase, and nitric oxide synthase, deprivation of arginine disrupts pleiotypic biochemical responses (28). With the deprivation of arginine, normal fibroblasts can survive in a G_0 state (10,29). However, tumor cells keep on dividing into apoptotic cell death (30). Tumor cells seem to be susceptible to arginine deprivation and die rather than arrest within the cell cycle, contrasting with the situation in normal cells.

We previously reported the biochemical characterization of recombinant ADI which originated from *M. arginini* and is expressed in *E. coli* (31,32), and found out that ADI has a potent anti-tumor activity for solid cancer cells and leukemic cells depending on ASS expression or activity (15,16,31). In the current study, ASS was expressed at a high level in both human retinoblastoma tissues and retinoblastoma cell lines, such as Y79 and SNUOT-Rb1. Retinoblastoma cell death, however, occurred with ADI treatment, which was similar to that of arginine deprivation from culture media (L-arg-free medium). These results indicate that ADI-induced retinoblastoma cell death could result from arginine deprivation, as retinoblastoma cells lack ASS activity even at high expression of ASS.

All these findings led us to conclude that arginine deprivation by ADI treatment could be another option of treatment for retinoblastoma due to the low ASS activity in retinoblastoma cells.

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