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Pegylated Arginine Deiminase (ADI-SS $PEG_{20,000\ mw}$) Inhibits Human Melanomas and Hepatocellular Carcinomas *in Vitro* and *in Vivo*

Charles Mark Ensor, Frederick W. Holtsberg, John S. Bomalaski, and Mike A. Clark[1]

Department of Biology, T. H. Morgan Building and Phoenix Pharmacologics, Inc., Advanced Science and Technology Commercialization Center, University of Kentucky, Lexington, Kentucky 40506

ABSTRACT

Some murine melanomas and hepatocellular carcinomas (HCCs) have been shown to be auxotrophic for arginine. Arginine deiminase (ADI; EC 3.5.3.6.), an arginine-degrading enzyme isolated from *Mycoplasma*, can inhibit growth of these tumors. We found that ADI was specific for arginine and did not degrade other amino acids. Although arginine is not an essential amino acid for most cells, all human melanomas and HCCs tested were found to be inhibited by ADI *in vitro*. Arginine is synthesized from citrulline in two steps by argininosuccinate synthetase and argininosuccinate lyase. Melanomas and HCCs did not express argininosuccinate synthetase mRNA but did express argininosuccinate lyase mRNA, suggesting that the arginine auxotrophy of these cells was a result of an inability to produce argininosuccinate synthetase. Human melanomas and HCCs were transfected with an expression plasmid containing argininosuccinate synthetase cDNA. The transfected cells were much more resistant to ADI than the parental cells *in vitro* and *in vivo*. Initial attempts to use ADI *in vivo* indicated that this enzyme had little efficacy, consistent with its short circulation half-life. Formulation of ADI with polyethylene glycol to produce ADI-SS $PEG_{20,000\ mw}$ resulted in an enzyme with a much longer circulation half-life that, and although equally effective *in vitro*, was more efficacious in the treatment of mice implanted with human melanomas and HCCs. These data indicate that sensitivity of melanoma and HCC is due to the absence of argininosuccinate synthetase in these cells and that an effective formulation of ADI, which causes a sustained decrease in arginine, may be a useful treatment for arginine auxotrophic tumors including melanoma and HCC.

INTRODUCTION

Arginine is a nonessential amino acid for humans and mice (1) because it can be synthesized from citrulline in two steps via the urea cycle enzymes argininosuccinate synthetase and argininosuccinate lyase. Argininosuccinate synthetase catalyzes the conversion of citrulline and aspartic acid to argininosuccinate. Argininosuccinate is then converted to arginine and fumaric acid by argininosuccinate lyase.

When a number of different types of murine tumor cells (in particular, melanomas and HCCs[2]) were found to be unable to synthesize arginine, it was suggested that an arginine-degrading enzyme may prove effective in inhibiting arginine-requiring cancers (2–7). Melanomas and HCCs can be killed *in vitro* by the addition of ADI to the culture medium and *in vivo* by injection of ADI into experimental animals. However, the *in vivo* use of ADI has two major disadvantages. First, ADI is only found in microorganisms and is strongly antigenic in mammals. Secondly, ADI has a short circulation half-life (~5 h) and must be frequently administered in large doses to inhibit tumors implanted into mice (2).

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[1] To whom requests for reprints should be addressed, at Phoenix Pharmacologics, Inc., Doctors Park, Suite 402, 1517 Nicholasville Road, Lexington, KY 40503. Phone: (859) 277-8890; Fax: (859) 277-8891; E-mail: mclarke@phoenixpharm.org.

[2] The abbreviations used are: HCC, hepatocellular carcinoma; ADI, arginine deiminase; RT-PCR, reverse transcription-PCR; SS-PEG, succinimidyl succinate polyethylene glycol; PEG, polyethylene glycol; SCID, severe combined immunodeficient; HPLC, high-performance liquid chromatography.

We set out to examine two hypotheses. The first was that ADI sensitivity and arginine auxotrophy of human melanoma and HCC cells were due to the lack of one or more of the urea cycle enzymes (specifically, argininosuccinate synthetase and argininosuccinate lyase) responsible for the biosynthesis of arginine. The second was that a PEG-formulated ADI (ADI-SS $PEG_{20,000\ mw}$), because of its increased circulation half-life, would be more effective than the native form of the enzyme for the treatment of arginine auxotrophic tumors *in vivo*.

To test these hypotheses, we examined the sensitivity of a large number of human melanoma and HCC cells to ADI and ADI-SS $PEG_{20,000\ mw}$ to determine the frequency of arginine auxotrophy in these tumor lines. Second, these cell lines were screened for the presence of argininosuccinate synthetase and argininosuccinate lyase mRNA by Northern blotting and RT-PCR to see whether the absence of one of these enzymes could explain arginine auxotrophy and ADI sensitivity. Selected ADI-sensitive, argininosuccinate synthetase mRNA-negative melanoma and HCC cells were transfected with an expression vector containing the human argininosuccinate synthetase coding sequence, and the cells were retested for sensitivity to ADI. Transfected cells were implanted into mice, and the mice were treated with native ADI and pegylated ADI. In addition, native ADI and pegylated ADI were compared by treating mice with implanted melanoma and HCC tumors. These results indicate that sensitivity of melanoma and HCC is due to the absence of argininosuccinate synthetase in these cells and that a PEG formulation of ADI that causes a sustained decrease in circulating arginine may be a useful treatment for arginine auxotrophic tumors.

MATERIALS AND METHODS

Materials. Alamar Blue dye was obtained from Alamar Biosciences, Inc. (Sacramento, CA). The plasmids containing the human argininosuccinate synthetase cDNA (pAS4/1/9) and AL1.4, which contains the human argininosuccinate lyase cDNA, were obtained from American Type Culture Collection (Manassas, VA). Expression plasmid pcDNA3 was from Invitrogen (San Diego, CA). All cell culture media were obtained from Life Technologies, Inc. (Rockville, MD), and FCS was from HyClone (Logan, UT). *O*-Phthalaldehyde, Thioflur, Li220, Li280, Li750, Regenerant, amino acid standards mixture, and Seraprep were purchased from Pickering Laboratories (Mountain View, CA). L-Arginine and citrulline were from Sigma (St. Louis, MO). Biotinylated goat antirabbit IgG, normal goat serum, Vectastain ABC kit, and 3,3′-diaminobenzidine were from Vector Laboratories (Burlingame, CA). The One-Step RT-PCR kit from Qiagen (Valencia, CA) was used for RT-PCR.

Production of ADI and ADI-SS $PEG_{20,000\ mw}$. Recombinant ADI was obtained by expression of the *Mycoplasma hominis* gene in *Escherichia coli* as described previously (8, 9). The specific activity of the purified ADI was 20 IU/mg protein. One IU of ADI enzyme activity was defined as the amount of enzyme activity that converts 1 μmol of arginine into 1 μmol of citrulline in 1 min at 37°C.

The purified enzyme was either used directly (native ADI) or formulated with PEG as follows. PEG was covalently attached to ADI using SS-PEG of M_r 20,000 (SS $PEG_{20,000\ mw}$) with the same methods used in formulating asparaginase with PEG (10) as described in detail in Ref. 8. The resulting PEG-formulated ADI was termed ADI-SS $PEG_{20,000\ mw}$.

The number of PEG molecules attached to the primary amines of ADI was determined exactly as described for PEG-asparaginase (10). Briefly, serial

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dilutions of the ADI native enzyme and ADI-SS $PEG_{20,000\ mw}$ were made in 100 mM sodium phosphate (pH 8.3). TNBS (2,4,6-Trinitrobenzene sulfonic acid) reagent was then added (10 mg/ml), and the reactions were heated to 40°C for 2 h. The absorbance was determined ($A_{330\ nm}$), and the absorbance *versus* the protein concentration was plotted, and a slope of the line was determined: the number of PEG molecules attached to each molecule of ADI = 1 − (slope of native ADI/slope of ADI-PEG) × 28 (28 represents the number of primary amines present in ADI). The resulting material, termed ADI-SS $PEG_{20,000\ mw}$, had 10–12 PEG molecules attached to each molecule of ADI and a specific enzyme activity of 12 IU/mg protein.

Cells and Cell Culture. All cell lines were obtained from American Type Culture Collection or Memorial Sloan Kettering Cancer Center (New York, NY). Cells were cultured in MEM with Earle's salts, supplemented with 10% (v/v) fetal bovine calf serum, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate unless otherwise indicated. In some experiments, the cells were grown in arginine-free MEM (MEM SELECT-AMINE), made according to the manufacturer's instructions, (Life Technologies, Inc.) supplemented with dialyzed fetal bovine serum [10% (v/v)], 1 mM sodium pyruvate, and 0 or 10 mM citrulline.

***In Vitro* Sensitivity of Tumor Cells to ADI.** Quantitative cell proliferation assays were performed using the Alamar Blue dye method according to the instructions provided by the manufacturer. Cells (2.5×10^3) in a volume of 0.1 ml of growth medium were added to each well of a 96-well plate. ADI was diluted into the growth medium (0.1 ml). The plates were incubated for 7 days at 37°C in an incubator containing an atmosphere of 95% air and 5% CO_2, and then 0.02 ml of Alamar Blue was added to each well, and the plates were incubated for an additional 5 h. The absorbance of the wells at 570 nm was then determined using a 96-well plate reader from Molecular Devices (Sunnyvale, CA). The amount of ADI needed to kill 50% of the cells in a culture was defined as the IC_{50}.

Northern Blotting and RT-PCR. Total RNA was isolated from human tumor cell lines grown in culture using guanidine isothiocyanate and standard laboratory methods. Ten μg of RNA from each cell line were separated by electrophoresis, transferred to nitrocellulose membranes, and then probed with a ^{32}P-labeled cDNA containing the full coding sequence. These blots were probed with argininosuccinate synthetase cDNA probe; stripped (by boiling in 1 mM EDTA) and rehybridized using an argininosuccinate lyase cDNA probe; and then stripped again, probed with human actin cDNA probe, and re-exposed to X-ray film.

For RT-PCR, the One-Step RT-PCR kit from Qiagen was used as suggested by the manufacturer. The primers used were as follows: forward primer, 5′-ccaaccgcgagaagatacccag-3′; and reverse primer, 5′-gggtacatggtggtgccgccagac-3′. These primers were chosen so that the yield from RNA would be a predicted 640-bp fragment and inadvertent amplification of DNA would result in the synthesis of a 1179-bp fragment as a result of two introns. The RT-PCR products were analyzed using a 2% agarose gel stained with ethidium bromide.

Transfection of Human Melanoma Cells with Constitutively Expressed Argininosuccinate Synthetase. ADI-sensitive cells were transfected with an expression plasmid containing the human argininosuccinate synthetase gene. The expression plasmid was constructed with the human cDNA encoding argininosuccinate synthetase under the regulation of a cytomegalovirus promoter. The plasmid also contained a neomycin resistance gene that allowed for selection of the melanoma cells that had been transfected. The *Pst*I fragment of the argininosuccinate synthetase cDNA from pAS4/1/9 was subcloned into the *Eco*RV site of pcDNA3. The resulting expression plasmid was linearized using *Pvu*I, and 10 μg were used to transfect 5×10^6 SK-mel-2, SK-mel-28, SK-hep 2, or SK-hep 3 cells by electroporation. Cells were plated out in 100-mm Petri dishes containing growth medium and allowed to grow for 48 h. G418 was then added (800 μg/ml) to the culture to kill cells that had not taken up the expression plasmid. After an additional 3 weeks of growth, isolated clones of the transfected cells were tested for the ability to grow in ADI.

Western Blotting. To demonstrate that transfected cells were expressing argininosuccinate synthetase protein, cells were stained using an anti-argininosuccinate synthetase polyclonal antibody. Recombinant human argininosuccinate synthetase was prepared by expressing this protein in *E. coli* using a vector (pET-22b+) that added six histidine amino acids to the COOH terminus of the protein (Novagen, Madison, WI). The recombinant protein was purified from bacteria using Ni affinity columns (Qiagen) as suggested by the manufacturer. The resulting recombinant argininosuccinate synthetase was injected into rabbits, and polyclonal antibodies were prepared. The anti-argininosuccinate synthetase antibodies were affinity purified using cyanogen bromide-coupled Sepharose affinity columns. Western blots were made using standard laboratory techniques, a secondary antibody (biotin-conjugated goat antirabbit IgG; Sigma), and Vectastain ABC Reagent as suggested by the manufacturer.

Mice. The nude and SCID mice used in this study were obtained from Charles River Laboratories (Wilmington, MA) and were females 2–4 months of age at the beginning of the study. All were provided with a diet of standard mouse chow and water *ad libitum*.

Implantation of Tumor Cells. The cells were removed from the tissue culture flasks with 0.05% trypsin and 0.05% EDTA in a balanced salt solution (Life Technologies, Inc.) and resuspended in growth medium. Approximately $1–5 \times 10^6$ cells were injected s.c. into the backs of the mice and allowed to grow to ~0.5 cm in diameter before treatment. The mice were injected with saline, native ADI, or ADI-SS $PEG_{20,000\ mw}$ as noted in the figure legends.

Amino Acid Analysis by HPLC. Amino acid analysis was performed using HPLC with a Pickering Laboratories PCX 5200 post-column derivitization instrument with the reactor temperature at 39°C and a fluorescence detector. This separation used a cation exchange column at 34°C. The column, standards buffers, and all other reagents were purchased from and used as suggested by Pickering Laboratories.

Measurement of the Pharmacodynamics of ADI and ADI-SS $PEG_{20,000\ mw}$. CD-1 mice (Charles River Laboratories; 5 mice/experimental group) were injected i.m. in the hind limb with ADI or ADI-PEG, as indicated. Blood was collected at the time points indicated in either EDTA or heparin and mixed with an equal volume of Seraprep (this reagent is strongly acidic and precipitates proteins, leaving free amino acids in the supernatant). The samples were centrifuged at 13,000 × *g* for 10 min, and the arginine and citrulline in the supernatant fraction were then separated by cation-exchange HPLC and quantified by a fluorescent detection method involving post-column derivatization with *O*-phthaldehyde and *N,N*-dimethylmercaptoethylamine, using reagents and methodologies suggested by the supplier (Pickering Laboratories). This chromatographic method requires only 1–2 μl plasma/assay.

Assay for Substrate Specificity. This assay was performed using two different assay conditions. The first used a mixture of 20 amino acid standards purchased from Pickering Laboratories. Twenty μl of the mixture (in which each of the amino acids was at a concentration of 20 mM) were mixed with 20 μl of 0.5 M sodium phosphate buffer (pH 7.2). Next, 1 μl of buffer, native ADI, or ADI-SS $PEG_{20,000\ mw}$ (50 IU/ml, final concentration) was added to each tube, which was then incubated at 37°C for 2 h. The reactions were terminated by the addition of an equal volume of Seraprep (Pickering Laboratories) to each tube. Samples (5 μl/injection) were analyzed by HPLC as described above.

The second assay used mouse plasma. Buffer, native ADI, or ADI-SS $PEG_{20,000\ mw}$ (50 IU/ml, final concentration in serum) was added to the plasma, and the reaction was incubated at 37°C for 2 h. The reactions were terminated by the addition of an equal volume of Seraprep, and 5-μl samples were analyzed by HPLC as described above.

RESULTS

Inhibition of Cultured Human Tumor Cells by ADI. A total of 23 human melanoma and 16 human HCC cell lines were tested for sensitivity to ADI inhibition (Table 1). All human melanoma and HCC cell lines were sensitive to inhibition by ADI *in vitro*. The ADI IC_{50}s for melanomas ranged from <0.01 to 0.3 μg/ml, whereas the ADI IC_{50}s for the HCC cell lines had values of <0.01 μg/ml. As controls, we used several other human tumor cell lines including a breast adenocarcinoma (T47-D), a lymphoma (MeWo), and a lung carcinoma (A549) that were all able to grow in the presence of 100 μg/ml, the highest concentration tested. The results using ADI-$PEG_{20,000\ mw}$ were essentially the same (Table 1), demonstrating that pegylation of the ADI did not adversely affect its antitumor activity *in vitro*.

ADI Amino Enzyme-degrading Specificity. Because other amino acid-degrading enzymes, such as asparaginase (11), can metabolize multiple amino acids, we set out to determine the substrate specificity

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Table 1 *ADI inhibition of human melanoma and HCC cells in vitro*

The cells listed were grown in 96-well plates for 24 h and then challenged with either 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, or 30 μg of ADI or ADI-SS $PEG_{20,000\ mw}$. After an additional 7 days, the viability of the cells was determined using Alamar Blue, and the IC_{50} was determined. In addition to the human melanoma and HCC cells shown, several other human tumor cell lines were tested by the same methodology, including a breast adenocarcinoma (T47-D), a lymphoma (MeWo), and a lung carcinoma (A549), all of which were able to grow in the presence of 100 μg/ml, the highest concentration tested.

Melanoma cell line	ADI IC_{50} (μg/ml)	ADI-SS $PEG_{20,000\ mw}$ IC_{50} (μg/ml)	HCC cell line	ADI IC_{50} (μg/ml)	ADI-SS $PEG_{20,000\ mw}$ IC_{50} (μg/ml)
SK-mel-2	<0.01	0.01	SK-hep 1	<0.01	0.01
SK-mel-3	<0.01	0.01	SK-hep 2	<0.01	0.01
SK-mel-28	0.01	0.01	SK-hep 3	<0.01	0.01
SK-mel-37	0.10	0.10	HB8065	<0.01	0.01
A375	0.1	0.10	HB8064	<0.01	0.01
HTB67	<0.01	0.01	CRL8024	0.03	0.03
HTB68	<0.01	0.01	CRL2238	0.03	0.03
HTB70	0.1	0.1	CRL2235	<0.01	0.01
HTB71	0.01	0.03	HTB52	<0.01	0.01
CRL1675	0.01	0.03	CRL2234	<0.01	0.01
CRL1678	0.10	0.1	CRL2237	<0.01	0.01
C32	0.01	0.01	HEP3B	<0.01	0.01
C32TG	0.01	0.01	CCL13	0.03	0.1
G361	0.1	0.1	HTB52	<0.01	0.01
HMCB	0.3	0.3	HEPG2	0.01	0.01
HS294T	0.3	0.3	WRL68	0.01	0.01

of native ADI and ADI-SS $PEG_{20,000\ mw}$. This was done using two different assays. The first used a mixture containing 20 purified amino acids, all present at the same concentration (100 μM; Table 2). The amino acid mixtures were then incubated with either native ADI or ADI-SS $PEG_{20,000\ mw}$. In the second assay, mouse plasma (obtained from normal mice by vena puncture) was incubated with either native ADI or ADI-SS $PEG_{20,000\ mw}$. Although both assays used a large excess of ADI, only the arginine (retention time, ~61 min) content was observed to diminish with both native and pegylated forms, and there was a corresponding increase in citrulline (retention time, ~28 min; Fig. 1 and Table 2). From these experiments, there is no evidence that ADI degrades any amino acid other than arginine.

Expression of Argininosuccinate Synthetase and Argininosuccinate Lyase mRNA. Previous investigators had suggested that melanomas may be sensitive to ADI as a result of their inability to express argininosuccinate synthetase (7). Because citrulline is converted into arginine by the sequential actions of argininosuccinate synthetase and argininosuccinate lyase, an inability to express either enzyme would be expected to result in arginine auxotrophy and sensitivity to ADI.

Table 2 *Specificity of ADI using a mixture of purified of amino acids as substrate*

ADI (50 IU/ml) was incubated with a mixture of 20 amino acids, all present in an equal molar concentration, for 2 h at 37°C, and then the proteins were precipitated with acid, and the amino acids were quantified using HPLC as shown in Fig. 2. The area under the curve of each amino acid was integrated and is shown.

Amino acid	Retention time (min)	Control area	+ ADI area
Asp	2.91	5803	5700
Thr	3.38	3591	3505
Ser	9.03	3665	3569
Asp	11.89	3601	3513
Gln	12.89	5928	5199
Glu	15.73	4366	4300
Pro	21.79	2814	2799
Gln	26.02	5687	5686
Ala	27.27	5076	4978
Cit	28.11	4858	9762[a]
Val	28.99	4856	4863
Met	30.17	3945	3890
Ilu	32.14	17431	17284
Leu	32.86	15650	15535
Tyr	33.73	9207	9133
Phe	35.41	4315	4290
H-Cys	48.93	20968	20752
Trp	49.97	6462	6373
Lys	56.30	5242	5246
His	59.44	2038	1980
Arg	61.28	5369	77[a]

[a] The only amino acid that appeared to be metabolized was arginine, and there was an increase in citrulline.

Because a possible defect in argininosuccinate lyase expression in the murine melanomas had not been excluded, we examined the expression of both of these enzymes by Northern blot analyses in human melanomas and HCCs as well as in a number of human ADI-resistant tumor cell types including breast adenocarcinoma (T47-D), lymphoma (MeWo), and lung carcinoma (A549). Total RNA was isolated from the cells indicated, and Northen blots were prepared. The blots were first probed using the argininosuccinate synthetase cDNA (Fig. 2*A*), then stripped and probed with argininosuccinate lyase cDNA (Fig. 2*B*), and then stripped again and probed with actin cDNA (Fig. 2*C*). Results from these experiments indicate that melanomas and HCCs do not produce detectable amounts of argininosuccinate synthetase mRNA but do produce argininosuccinate lyase mRNA. In many instances, the levels of argininosuccinate lyase mRNA appeared to be higher in argininosuccinate synthetase-deficient cell lines than in those cells found to contain argininosuccinate synthetase mRNA. The reason for this is not clear at the present time. Only the melanoma and HCC cell lines, which lack argininosuccinate synthetase mRNA, were sensitive to killing by ADI; the breast adenocarcinoma (T47-D), lymphoma (MeWo), and lung carcinoma (A549) cell lines, which express argininosuccinate synthetase, were all resistant to ADI and were able to grow even in the presence of large amounts (100 μg/ml) of ADI.

Transfection of Melanoma and HCC Cells with Argininosuccinate Synthetase Results in Resistance to ADI. To confirm that it was an inability to produce argininosuccinate synthetase that conferred ADI sensitivity on cells, the human melanoma cell lines SK-mel-2 and SK-mel-28 and HCC cell lines SK-hep 2 and SK-hep 3 were transfected with an expression plasmid containing human argininosuccinate synthetase cDNA. RT-PCR analysis using primers for argininosuccinate synthetase indicated the presence of argininosuccinate synthetase RNA that was not seen in ADI-sensitive untransfected cells but was clearly present in the transfected cells (Fig. 3). These cells were then analyzed for sensitivity to ADI treatment. It was found that the cells transfected with the argininosuccinate synthetase gene were at least 1000 times more resistant to ADI than the untransfected parental cells (Fig. 4).

Pharmacodynamic Effects of ADI and ADI-SS $PEG_{20,000\ mw}$ on Plasma Arginine Levels in Mice. The effects of injection of ADI and ADI-SS $PEG_{20,000\ mw}$ on the plasma levels of arginine (the pharmacodynamics) were determined by administering ADI and ADI-SS $PEG_{20,000\ mw}$ (5 IU/mouse, i.m.) to mice. The plasma was collected at the indicated times, and the levels of arginine were

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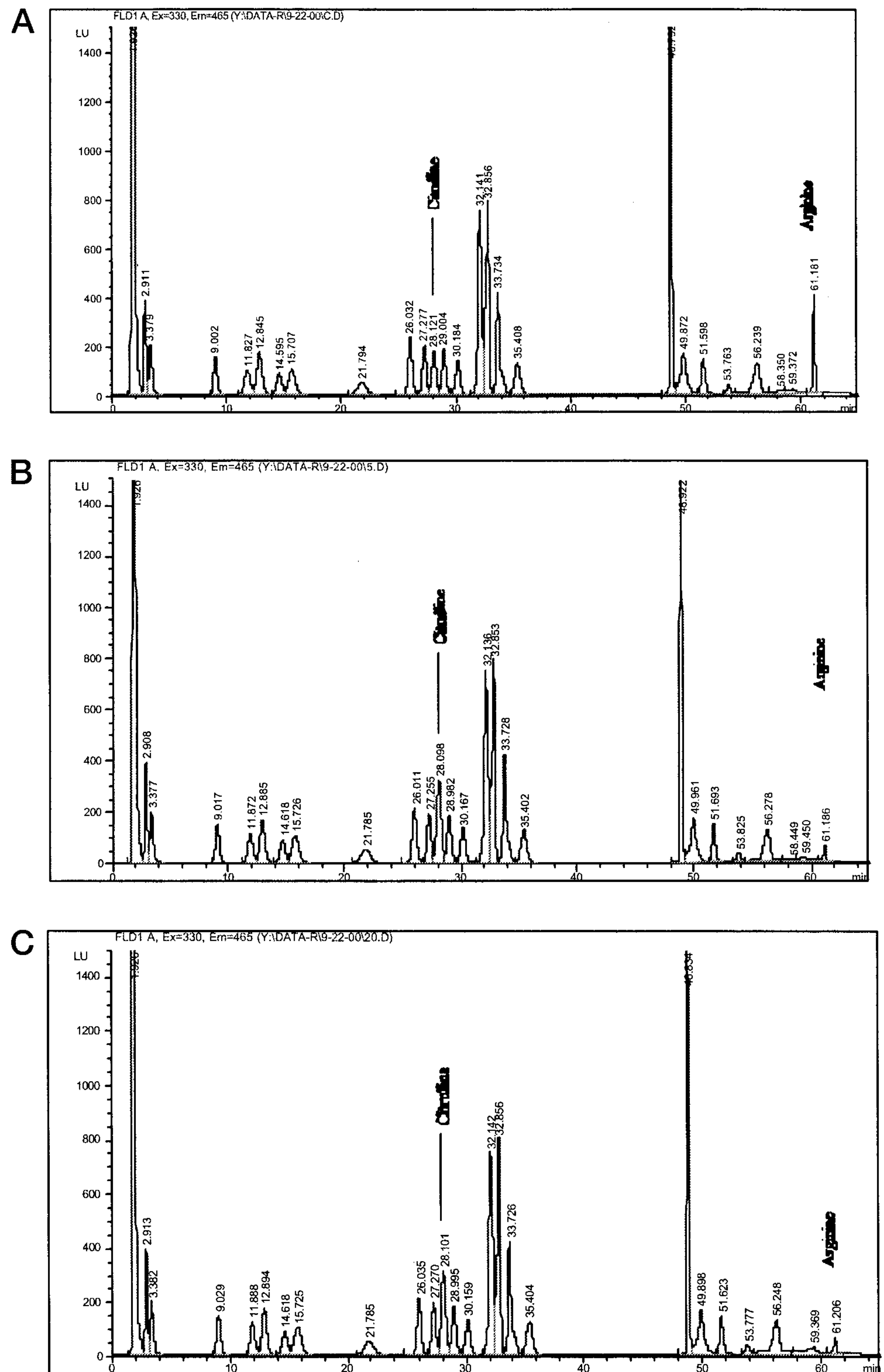

Fig. 1. Substrate specificity of ADI and ADI-SS $PEG_{20,000\ mw}$. Normal mouse plasma was incubated with buffer (*A*), native ADI (50 IU/ml; *B*), or ADI-SS $PEG_{20,000\ mw}$ (50 IU/ml; *C*) for 2 h at 37°C. After incubation, the protein was precipitated, and amino acid analysis of the supernatant was performed by HPLC as described in "Materials and Methods."

determined by amino acid analysis. The results are shown in Fig. 5. This dose of ADI-SS $PEG_{20,000\ mw}$ reduces circulating arginine levels in mice to below detectable levels for ~7 days. The same dose of native ADI (5 IU/mouse, i.m.) will reduce arginine by only about 40–50% at 24 h after injection, with arginine levels returning to normal by 48 h postinjection.

Effects of ADI and ADI-SS $PEG_{20,000\ mw}$ on Human Melanomas and HCC Implanted into Mice. To test the effectiveness of native ADI and ADI-SS $PEG_{20,000\ mw}$ in inhibiting melanomas and HCCs *in vivo*, mouse xenograph models were used. Human melanoma cell lines SK-mel-2 and SK-mel-28 were implanted into immunodeficient mice and allowed to grow to ~0.5 cm in size. The animals then received injection with saline or 5 IU of ADI twice a week for 2 weeks or with 5 IU of ADI-SS $PEG_{20,000\ mw}$ once a week for 2 weeks, and the effects on tumor growth noted. It was observed that native ADI had no appreciable antitumor activity against either melanoma *in vivo* because the sizes of the tumors continued to increase at essentially the same rate as in saline-treated animals (Fig. 6). These results

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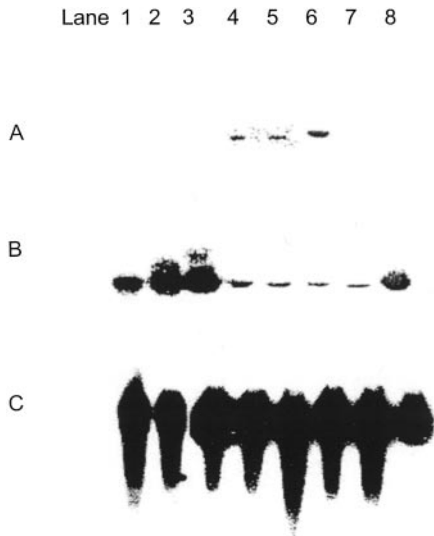

Fig. 2. Northern blots of RNA isolated from human melanomas and HCC. The lanes in both blots contain mRNA from the following cell lines: *Lane 1,* SK-mel-2 melanoma; *Lane 2,* SK-mel-3 melanoma; *Lane 3,* SK-mel-28 melanoma; *Lane 4,* MeWo lymphoma; *Lane 5,* T47-D breast adenocarcinoma; *Lane 6,* A549 lung carcinoma; *Lane 7,* SK-hep 2 HCC; and *Lane 8,* SK-hep 3 HCC. The blot was initially probed with human cDNA encoding argininosuccinate synthetase cDNA (*A*). This same blot was then stripped and probed with argininosuccinate lyase probe (*B*) and then stripped and probed using human actin cDNA (*C*).

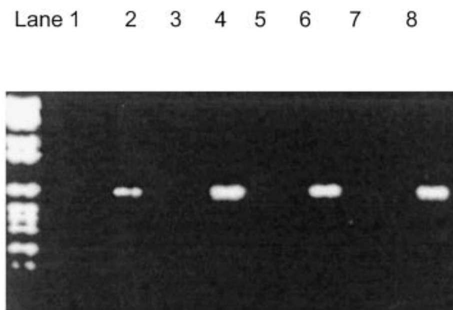

Fig. 3. Expression of argininosuccinate synthetase mRNA in human melanoma and HCC cells transfected with human cDNA encoding argininosuccinate synthetase. Human melanoma cell lines used were SK-mel-2 (*Lanes 1* and *2*) SK-mel-28 (*Lanes 3* and *4*), and human HCC cell lines used were SK-hep 2 (*Lanes 5* and *6*) and SK-hep 3 (*Lanes 7* and *8*). Each of these cell lines was transfected with an expression plasmid containing human argininosuccinate synthetase. RNA was isolated from the parental cells (*Lanes 1, 3, 5,* and *7*) and transfected cells (*Lanes 2, 4, 6,* and *8*). RT-PCR was used to determine whether argininosuccinate synthetase mRNA was being produced.

were consistent with the effects these two formulations of ADI have on plasma arginine (shown in Fig. 5).

Likewise, native ADI had little effect on animal survival compared with saline-treated animals implanted with HCC tumors (Fig. 7). ADI-SS $PEG_{20,000\ mw}$ greatly increased the survival rate of tumor-bearing mice, with 50% or more of the treated mice surviving to 24 weeks. In contrast, the saline- and native ADI-treated mice all died within 7 weeks.

In another experiment, the human melanoma cell lines SK-mel-2 and SK-mel-28 and HCC cell lines SK-hep 2 and SK-hep 3, which had been transfected with the human argininosuccinate synthetase cDNA and were resistant to ADI *in vitro* (Fig. 4), were assessed for their responses to ADI-SS $PEG_{20,000\ mw}$ *in vivo*. Mice were implanted with the transfected melanoma and HCC cells described above in Figs. 3 and 4. Once the tumors had attained a size of ~0.5 cm in diameter, the animals were treated with saline or ADI-SS $PEG_{20,000\ mw}$ (5 IU/mouse, i.m.). Tumors resulting from implantation of argininosuccinate synthetase-transfected cells expressed argininosuccinate synthetase as determined by Western blot analysis (Fig. 8) and were also resistant to ADI-SS $PEG_{20,000\ mw}$ *in vivo*, even when this drug was administered at a dose of 5 IU/mouse daily for 2 weeks (Fig. 9).

DISCUSSION

Influenced by the success of asparaginase in the clinic (12, 13), J. B. Jones (14) first used ADI purified from *Pseudomonas pudita* as an anticancer therapy. Although this enzyme inhibited tumor cells *in vitro*, no appreciable activity was observed *in vivo*. It was Takaku *et al.* (2–5) who first used ADI isolated from *Mycoplasma* and found that this enzyme was active *in vitro* and *in vivo*, although large doses had to be administered frequently *in vivo* for significant antitumor efficacy to be observed. Takaku *et al.* (2–5) explained the lack of success of

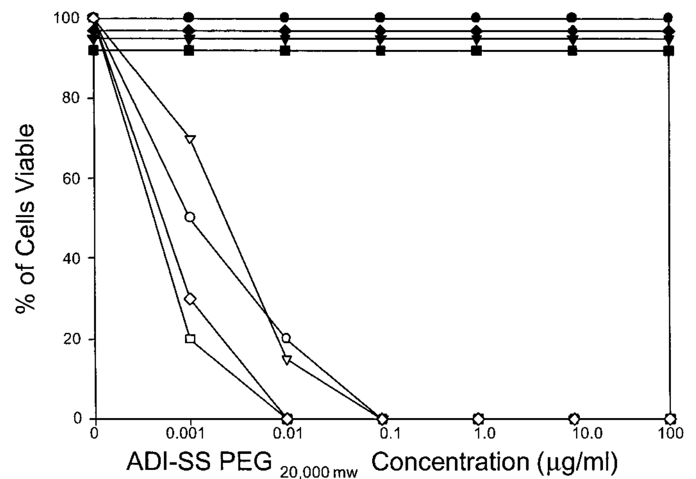

Fig. 4. Transfection of human melanoma cells with human cDNA encoding argininosuccinate synthetase confers resistance to ADI. Human melanoma cell lines SK-mel-2 (▽, ▼) and SK-mel-28 (●, ○) and HCC cell lines SK-hep 2 (◇, ◆) and SK-hep 3 (□, ■) were transfected with an expression plasmid composed of human cDNA encoding argininosuccinate synthetase under the constitutive expression of a cytomegalovirus promoter. The transfected cells (*solid symbols*) and the parental cells (*open symbols*) were grown in 96-well plates and incubated with the indicated concentrations of ADI-SS $PEG_{20,000\ mw}$. After 7 days, the cells were stained using a vital dye, and the percentage of living cells was recorded. The data shown are the means of quadruplicate cultures of cells. All of the transfected cells retained 100% viability at all concentrations of ADI tested; the *lines* in the figure have been separated for clarity.

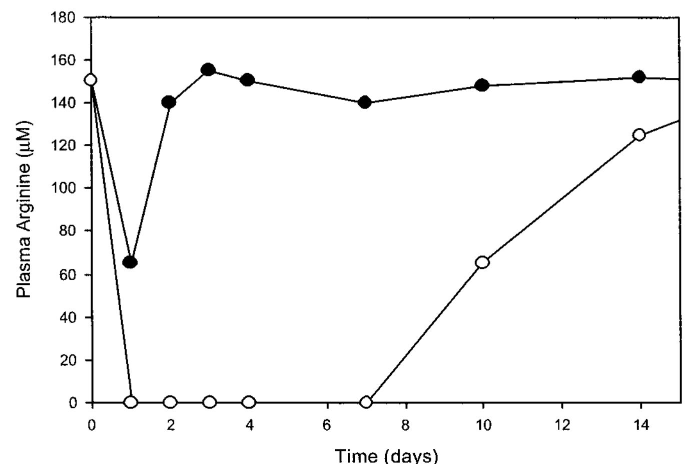

Fig. 5. The pharmacodynamics of ADI and ADI-SS $PEG_{20,000\ mw}$ on plasma arginine in mice. CD-1 mice (5 female animals/group) were each injected i.m. with 5 IU of either ADI (●) or ADI-SS $PEG_{20,000\ mw}$ (○). Plasma was collected at the indicated times, and the amount of arginine in each sample was determined by amino acid analysis. The data shown represent the means of the five animals. The SE between animals was <11%; the intra- and interassay variations were 6% and 7%, respectively.

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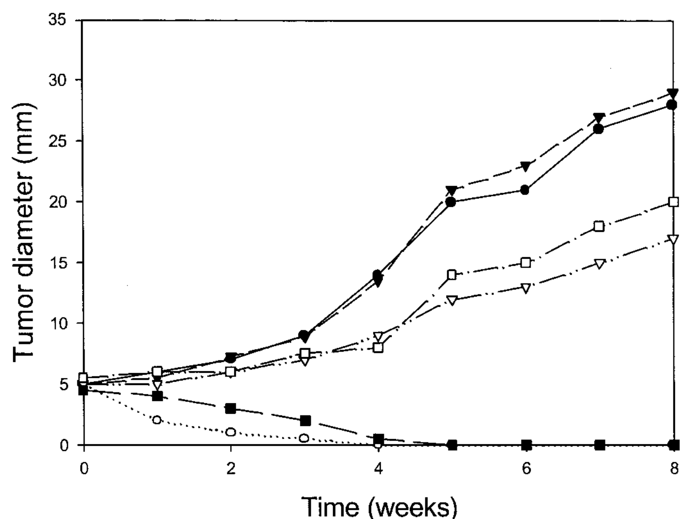

Fig. 6. The effects of ADI and ADI-SS $PEG_{20,000\ mw}$ on the growth of human melanomas implanted into nude mice. Athymic mice were implanted with SK-mel-2 and SK-mel-28 human melanomas. Approximately 10^6 cells were injected s.c. into each mouse, and the tumors were allowed to grow until they reached approximately 0.5 cm in diameter. The animals were then randomly assigned into groups (10 animals were implanted with each tumor in each treatment group). Mice were then treated twice a week for 2 weeks with either saline (▼, SK-mel-2; □, SK-mel-28) or 5 IU of ADI (●, SK-mel-2; ▽, SK-mel-28) or once a week with 5 IU of ADI-SS $PEG_{20,000\ mw}$ (○, SK-mel 2; ■, SK-mel 28). The size of the tumors was measured once a week, and the results are shown.

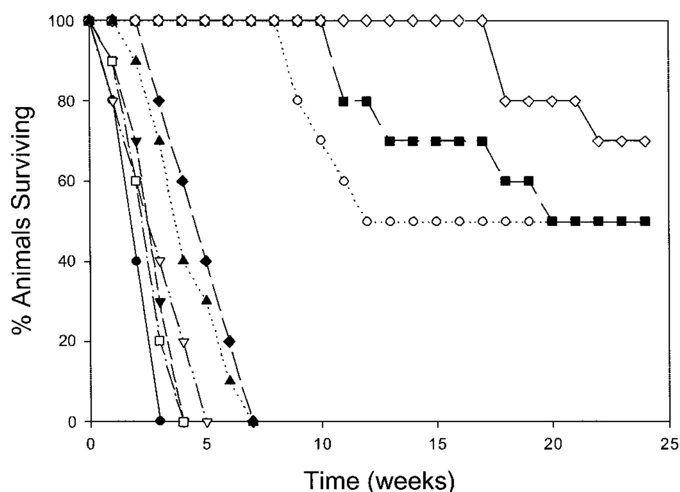

Fig. 7. The effects of ADI and ADI-SS $PEG_{20,000\ mw}$ on the survival of SCID mice implanted with HCCs. SCID mice were implanted with the human HCC cell lines SK-hep 1, SK-hep 2, and SK-hep 3 (30 mice were implanted with each tumor). Approximately 10^6 cells were injected s.c. into each mouse, and the tumors were allowed to grow until they reached approximately 0.5 cm in diameter. The mice were then divided randomly into groups (10 animals in each treatment group) and then treated i.m. with either saline (●, SK-hep 1; □, SK-hep 2; ♦, SK-hep 3), 5 IU of ADI (▼, SK-hep 1; ▲, SK-hep 2; ▽, SK-hep 3), or 5 IU of ADI-SS $PEG_{20,000\ mw}$ (○, SK-hep 1; ■, SK-hep 2; ◇, SK-hep 3). The treatments were given once a week for 2 weeks, and then the survival (percentage of animals alive each week) was recorded and is shown above.

Jones (14) on the pH optimum of the enzyme used. ADI isolated from *P. pudita* has an acidic pH optimum and is largely inactive at physiological pH, whereas the *Mycoplasma* enzyme has an optimum nearer to physiological pH. Sugimura *et al.* (6, 7) showed that five melanomas were sensitive to arginine deprivation therapy and thus defined this disease as a target for this therapy.

Because these previous investigators used a limited number of mouse cell lines, we set out to determine the relative incidence of arginine auxotrophy in a large number of human melanomas and HCCs. We focused primarily on these tumors, in part because of the work by earlier investigators, but also because of the observation that levels of free arginine are increased 5–20-fold in several HCCs (15). Furthermore, the urea cycle is depressed in most HCCs, and these cells must obtain arginine from the circulation. Therefore, depletion of plasma arginine would be expected to result in growth inhibition of these tumors *in vivo* (for review, see Ref. 14). We were surprised that all of the human melanomas and HCCs tested were sensitive to ADI inhibition *in vitro*.

Sugimura *et al.* (7) examined the RNA from several melanomas and found that the argininosuccinate synthetase message did not appear to be produced. They hypothesized that it was a lack of this enzyme that was responsible for the arginine auxotrophy of these cells. We extended this work and examined not only the expression of argininosuccinate synthetase but also that of argininosuccinate lyase. Consistent with the observations of Sugimura *et al.* (7), we were unable to detect argininosuccinate synthetase mRNA but were able to detect argininosuccinate lyase mRNA in all melanoma and HCC cell lines we tested. All cell lines found to be sensitive to ADI did not produce any detectable argininosuccinate synthetase. Whereas all argininosuccinate synthetase-negative melanomas and HCCs were much more sensitive to ADI than argininosuccinate synthetase-positive cell lines, there is a relatively wide range of sensitivity to ADI exhibited by these

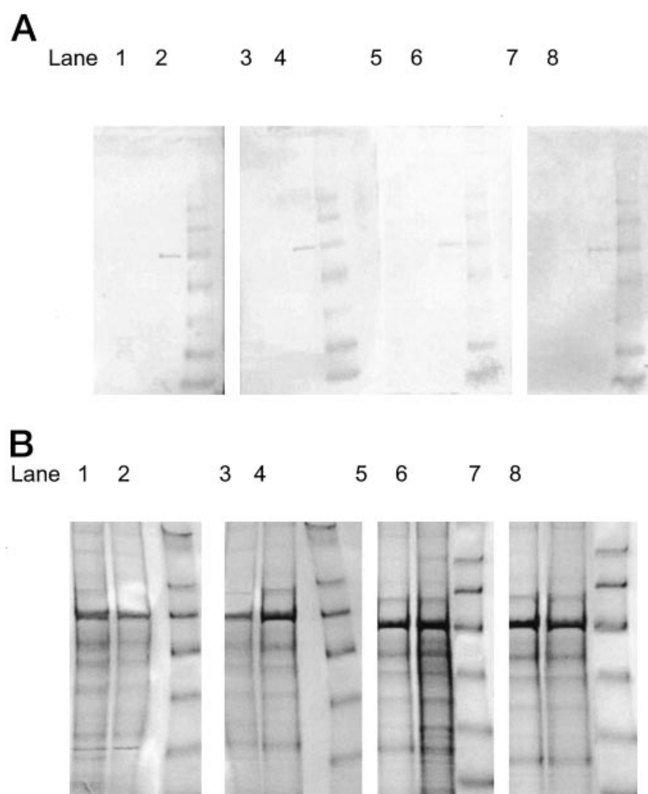

Fig. 8. Western blot analysis of human xenograph tumors. The transfected cells characterized for their sensitivity to arginine deprivation as shown above in Fig. 4 were also tested for expression of argininosuccinate synthetase *in vivo*. Human melanoma cell lines used were SK-mel-2 (*Lanes 1* and *2*) SK-mel-28 (*Lanes 3* and *4*), and human HCC cell lines used were SK-hep 2 (*Lanes 5* and *6*) and SK-hep 3 (*Lanes 7* and *8*). Approximately 1 million cells of each of the cell lines were injected into nude mice. Tumors were grown to ~0.5 cm in diameter, excised, minced, and finally sonicated in sample buffer. The cell-free extracts were separated using SDS-PAGE, and the proteins were then transferred to membranes and probed with affinity-purified antihuman argininosuccinate synthetase antibody. Extracts from tumors resulting from the parental cells (*Lanes 1, 3, 5,* and *7*) and extracts from tumor resulting from the transfected cells (*Lanes 2, 4, 6,* and *8*) were analyzed by Western blot using anti-argininosuccinate synthetase antibody (*A*). Extracts from tumors resulting from the parental cells (*Lanes 1, 3, 5,* and *7*) and extracts from tumors resulting from the transfected cells (*Lanes 2, 4, 6,* and *8*) were analyzed by Coomassie Blue-stained gels to determine the loading of protein extracts from the parental cells and the transfected cells (*B*).

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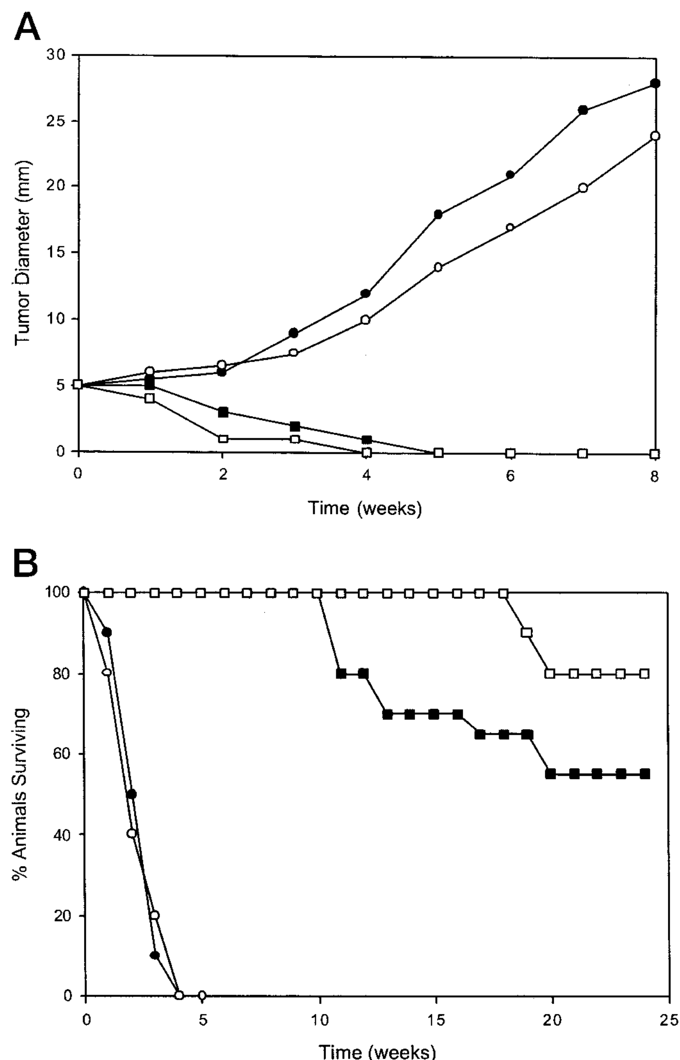

Fig. 9. Human xenograph tumors expressing argininosuccinate synthetase are resistant to inhibition by ADI-SS $PEG_{20,000\ mw}$. The transfected human melanoma cell lines SK-mel-2 (●) and SK-mel-28 (○) and the parental cell lines transfected with the expression vector minus human cDNA encoding argininosuccinate synthetase (SK-mel-2, ■; SK-mel-28, □) were injected into nude or SCID mice as described in Figs. 6 and 7 and allowed to produce tumors of ~0.5 cm in diameter. The mice were then treated once a week for 2 weeks with 5 IU of ADI-SS $PEG_{20,000\ mw}$. The tumors resulting from implantation of melanomas were measured once a week, and the results are shown in *A*. The transfected human HCC cell lines SK-hep 2 (●) and SK-hep 3 (○) and the parental cell lines transfected with the expression vector minus human cDNA encoding argininosuccinate synthetase (SK-hep 2, ■; SK-hep 3, □) were injected into nude or SCID mice. The mice were then treated once a week for 2 weeks with 5 IU of ADI-SS $PEG_{20,000\ mw}$. Survival of mice implanted with HCC tumors was also determined (*B*).

cells, with IC_{50}s ranging from <0.01 to 0.3 μg/ml. At present, we do not know the reason for these differences in sensitivity.

To confirm that it was a deficiency of argininosuccinate synthetase that caused sensitivity to ADI treatment, argininosuccinate synthetase mRNA-deficient cells were transfected with an expression plasmid containing argininosuccinate synthetase cDNA. It was found that the transfected cells, which now produce argininosuccinate synthetase protein as determined by immunostaining, were much more resistant to ADI than the parental cells *in vitro* and *in vivo*.

Initial attempts to use native *Mycoplasma* ADI as an anticancer treatment *in vivo* were of limited success because large amounts of ADI had to be administered daily to achieve an appreciable effect on tumor growth *in vivo*. It was hypothesized this failure was a result of the short circulation half-life of ADI (~5 h) and the consequent quick return to normal of plasma levels of arginine. We have found that the pegylation increases the half-life of ADI to ~7 days in mice and, at a dose of 5 IU once a week, will deplete serum arginine to below detectable levels for 6–8 days (8). Although ADI and ADI-SS $PEG_{20,000\ mw}$ were similar in their ability to inhibit the growth of melanomas and HCCs *in vitro* (Table 1), only the ADI-SS $PEG_{20,000\ mw}$ was found to be effective in inhibiting the growth of these tumor cells *in vivo*. It would appear that to achieve efficacy *in vivo*, serum arginine levels must be maintained at low levels for some time. This observation was supported by *in vitro* experiments in which melanomas and HCCs were incubated in medium without arginine or citrulline. Under these conditions, most of the cells exclude trypan blue for up to 5 or 7 days. If the cells are given complete medium before that time, a significant number of the cells are able to begin growing again (data not shown). Thus, for native ADI to be efficacious, it must either be given daily in large doses for several weeks or formulated so that it circulates longer. Thus, the shortcomings of the native enzyme are overcome by formulation of ADI with $PEG_{20,000\ mw}$ to produce what we have termed ADI-SS $PEG_{20,000\ mw}$, as shown by the dramatic effects on tumor size *in vivo* and animal survival rate.

Asparaginase, which is currently used in the treatment of acute lymphoblastic leukemia, degrades both asparagine and glutamine (11). It was recently shown that the antitumor activity of this enzyme is due to its ability to degrade asparagines, and some of its deleterious side effects were due to its degradation of glutamine (16). In contrast, ADI appears to degrade only arginine and does not appear to metabolize any other amino acids.

We have yet to see resistance to ADI develop in cultured cells, although we continue to look for resistant cells because these cells could give us important clues as to why melanomas and HCCs do not produce argininosuccinate synthetase. At this time, it is not known why human melanomas and HCCs are unable to express argininosuccinate synthetase. Initial examination of the gene indicated no obvious defect in its structure or the promoter region. At present, the molecular defect is not known and is a focus of research in this laboratory.

More recent investigations have confirmed that ADI inhibits cell proliferation (17). ADI was more potent at inhibiting human leukemia cells than asparaginase (18). Another group confirmed earlier studies, demonstrating that arginine depletion from culture medium resulted in rapid and selective death of transformed and malignant cells (19). These investigators have confirmed the importance of arginine and ADI in the auxotrophy of selected tumors. Thus, there is considerable evidence that ADI, especially ADI formulated with PEG (ADI-SS $PEG_{20,000\ mw}$), may have utility as a treatment for arginine auxotrophic tumors such as melanomas and HCCs. This compound may also be useful in additional tumors that are found to be argininosuccinate synthetase deficient or auxotrophic for arginine due to reasons other than argininosuccinate synthetase deficiency. ADI-SS $PEG_{20,000\ mw}$ is currently being tested in humans with metastatic melanoma and HCC.

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