

In vivo Loss of Expression of Argininosuccinate Synthetase in Malignant Pleural Mesothelioma Is a Biomarker for Susceptibility to Arginine Depletion

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Abstract Purpose: Malignant pleural mesothelioma (MPM) is an increasing health burden on many societies worldwide and, being generally resistant to conventional treatment, has a poor prognosis with a median survival of <1 year. Novel therapies based on the biology of this tumor seek to activate a proapoptotic cellular pathway. In this study, we investigated the expression and biological significance of argininosuccinate synthetase (AS), a rate-limiting enzyme in arginine production.

Experimental Design: Initially, we documented down-regulation of AS mRNA in three of seven MPM cell lines and absence of AS protein in four of seven MPM cell lines. We confirmed that the 9q34 locus, the site of the AS gene, was intact using a 1-Mb comparative genomic hybridization array; however, there was aberrant promoter CpG methylation in cell lines lacking AS expression, consistent with epigenetic regulation of transcription. To investigate the use of AS negativity as a therapeutic target, arginine was removed from the culture medium of the MPM cell lines.

Results: In keeping with the cell line data, 63% (52 of 82) of patients had tumors displaying reduced or absent AS protein, as assessed using a tissue microarray. Cell viability declined markedly in the AS-negative cell lines 2591 and MSTO but not in the AS-positive cell line, 28. This response was apparent by day 4 and maintained by day 9 *in vitro*. Arginine depletion induced BAX conformation change and mitochondrial inner membrane depolarization selectively in AS-negative MPM cells.

Conclusions: In summary, we have identified AS negativity as a frequent event in MPM *in vivo*, leading to susceptibility to cytotoxicity following restriction of arginine. A phase II clinical trial is planned to evaluate the role of arginine depletion in patients with AS-negative MPM.

The asbestos-related disease, malignant pleural mesothelioma (MPM), remains for the majority of patients an incurable disease with a median survival of 9 to 12 months from presentation (1). The incidence of MPM is projected to increase

over the next 10 to 20 years, in part, due to the long natural history of the disease as well as continued asbestos exposure in many parts of the world (2). Current approaches to therapy for this disease are limited. A new paradigm is clearly needed to move beyond the therapeutic plateau of chemotherapy regimens, such as pemetrexed and cisplatin, and the infrequently done surgical procedure of extrapleural pneumonectomy (3).

Increasingly, an enhanced knowledge of the molecular defects underlying tumorigenesis is being translated into effective targeted therapies for patients with cancer. These include specific growth receptor antagonists, antiangiogenic compounds, and drugs that exploit aberrant core-apoptosis signaling (4). Here, we have focussed on the role of tumor metabolism in MPM by exploring the relationship between the amino acid arginine and its biosynthetic rate-limiting enzyme, argininosuccinate synthetase (AS). Arginine is a precursor for a variety of molecules with important roles in tumorigenesis, including nitric oxide, nucleotides, proline, and polyamines (5–11). Arginine, for instance, may be critical to the free radical damage that accompanies the chronic inflammation of asbestos-induced MPM (12–14). Although a nonessential amino acid for normal cells due to the ubiquitous expression of AS, arginine becomes semi-essential during times of stress, such as burns or sepsis (15). Based on previous reports in

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hepatocellular carcinoma and melanoma, tumors that are arginine auxotrophs due to an intrinsic lack of AS and thus sensitive to arginine depletion (16), we assessed expression of this enzyme in MPM. In this study, we show that MPM is characterized by significantly decreased expression of AS. In particular, AS-negative MPM may be sensitized to arginine withdrawal, undergoing death by apoptosis.

Materials and Methods

Cell lines and growth conditions. Seven MPM cell lines (28, 226, 2052, and MSTO from the American Type Culture Collection (Middlesex, United Kingdom) and 2591, 2452, and 2461, a kind gift from Dr. Pasi Jänne of Dana-Farber Cancer Institute, Boston, MA) were grown in a humidified atmosphere at 37°C and 5% CO₂ in endotoxin-free RPMI 1640 supplemented with 10% fetal bovine serum (Sigma, Poole, United Kingdom). Cells at 70% confluency were harvested for analysis of constitutive AS mRNA and protein. Arginine depletion experiments (see below) were done using 2% dialyzed fetal bovine serum (>10 kDa; Autogen Bioclear, Wilts, United Kingdom).

Quantitative real-time reverse transcription-PCR. DNase-treated RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, Southampton, United Kingdom) according to the manufacturer's instructions. AS (FAM) primers and probes were designed using Primer Express version 1.5a software: AS, CAAGCGCTCCAGGTCTCTA (forward); GGACCCCTTTTGAAGCTCGAT (reverse); and AGACCCAGGACCCAGCCAAAGCC (probe). Multiplex real-time reverse transcription-PCR analyses were done using AS (FAM) and 18S rRNA (VIC) primers and probes with the ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, GB). Two microliter of cDNA were used per 25 μ l reaction with the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each sample was analyzed in triplicate and normalized (ΔC_t) to 18S by removing the cycle threshold (C_t) value of 18S from the C_t value of the AS gene. The ΔC_t for the positive control (IGROV-1) was subtracted from the ΔC_t for the remaining cell lines (i.e., $\Delta\Delta C_t$) and the fold difference was calculated by $2^{-\Delta\Delta C_t}$.

Comparative genomic hybridization array. DNA extraction from the MPM cells (*t* test) was followed by *Eco*R1 digestion and random primer labeling with Cy5 and Cy5 (Invitrogen, Paisley, United Kingdom). Complementary random primer labeling of sex-matched reference (R)

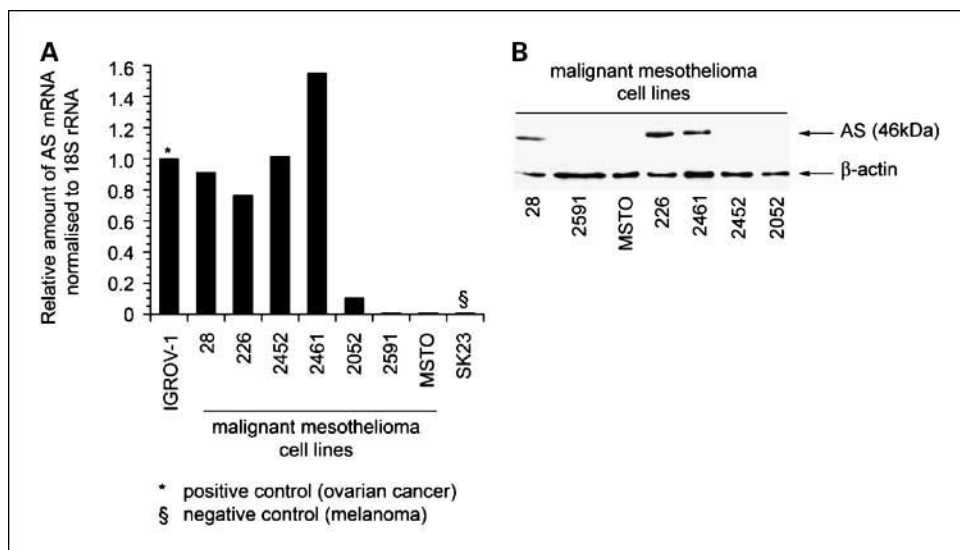
DNA with Cy5 and Cy3 was also conducted. Equal volumes of test and control DNA were combined and coprecipitated using NaCl and isopropanol. After an ethanol wash, samples were resuspended in water and hybridization buffer. Specimens were denatured and added to Spectral Genomics 2600 1-Mb comparative genomic hybridization (CGH) array (Stretton Scientific, Stretton, United Kingdom). The arrays were placed in a hybridization chamber containing 10 mL of 2 \times SSC/50% formamide, wrapped in aluminum foil, and sealed before 16 hours of incubation at 37°C followed by brief rinsing in 2 \times SSC, 0.5% SDS. The arrays were then washed in 2 \times SSC/50% formamide and 2 \times SSC/0.1% NP40, both for 20 minutes at 50°C. The final wash was for 10 minutes in 0.2 \times SSC, still at 50°C. Slides were then briefly washed in water and dried by using forced air. Hybridized slides were scanned with the GenePix 4000A scanner (Axon, Berkshire, United Kingdom) and the acquired images were analyzed using GenePix Pro4.0. For each cell line, two arrays were used and the data were normalized to 1.0. After analysis, data from the paired arrays were exported into Spectralware software to generate ratio plots.

Methylation-specific PCR. One microgram of genomic DNA was modified with sodium bisulphite using the EZ DNA methylation kit (Zymo, Cambridge, United Kingdom) according to the manufacturer's instructions. Fifty nanogram of modified DNA were amplified using the following primers designed using the AS promoter sequence (5): 5'TTTGATGTTTAAATGTTTGGTATTG (UF), 5'AATCCAAAAAACC-AATCCAAAAAACCCTACTACAC (UR), 5'TTCGATGTTTAAACGT-TTCGATGTTTAAACGTTTGGTATA (MF), and 5'CCAAAAAACC-ACCCGACTACG (MR), where U is unmethylated and M is methylated.

Western blot analysis. Whole-cell extracts were made from 70% confluent cultures of MPM cells, using 1% SDS Western lysis buffer. Cell extract (10 μ g) was run on a SDS-12% acrylamide gel and transferred to a nylon membrane. The membrane was blocked overnight [4°C in PBS with 0.1% Tween (PBST) and 10% milk powder] and probed using an anti-AS antibody at 1:2,500 (BD Biosciences, Oxford, United Kingdom) in 0.1% Tween (PBST) and 10% milk powder at room temperature for 1 hour. After washing with 0.1% Tween (PBST), the membrane was incubated in 0.1% Tween (PBST) and 10% milk powder with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution, room temperature for 1 hour). The secondary antibody was detected using the Western Lightning Chemiluminescence kit (Perkin-Elmer Life Sciences, Beaconsfield, United Kingdom).

AS immunohistochemistry. A tissue microarray block containing 82 of 99 evaluable MPM biopsies from patients treated in St. Bartholomew's Hospital (London, United Kingdom) was used. All

Fig. 1. AS mRNA and protein expression in malignant mesothelioma cell lines. **A**, RNA was obtained from 70% confluent cells using the ABI Prism 6100 Nucleic Acid PrepStation, reverse transcribed, and then analyzed for AS mRNA expression using quantitative real-time reverse transcription-PCR. A positive control (IGROV-1, ovarian cancer cell line) and a negative control (SK-23, melanoma cell line) were used to compare AS mRNA expression to that of the malignant mesothelioma cell lines. **B**, malignant mesothelioma cell line lysates were prepared as described in Materials and Methods and analyzed (10 μ g/lane) by Western blotting for expression of AS protein. AS was identified as a single band of 46 kDa, with β -actin representing the loading control.



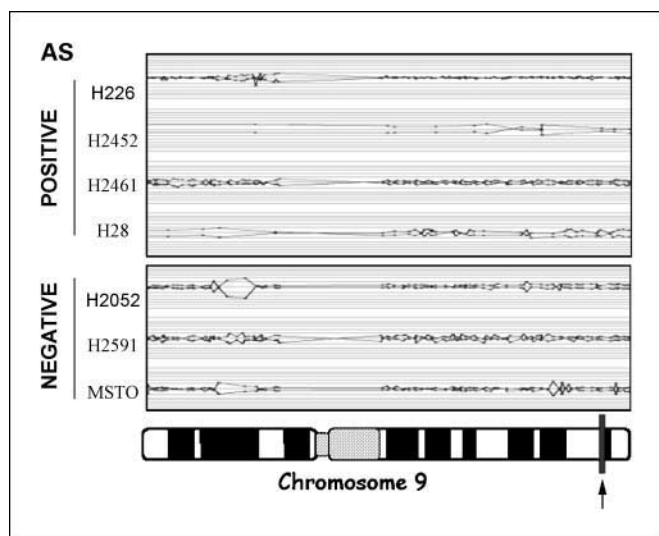


Fig. 2. CGH array. The 9q34.11 locus for the AS gene was intact in all seven mesothelioma cell lines, with no difference between the AS-negative and AS-positive cell lines (arrow).

patients gave informed consent for biopsy and tumor analysis. Confirmation of the diagnosis of MPM was obtained using a routine panel of immunohistochemical stains: DPAS, CK5/6, calretinin, EMA, CEA, and BerEP4. Cases that were consistently positive were then selected for the tissue microarray. Three-micron thick slides were cut and mounted on coated slides. After overnight incubation at 40°C, the slides were dewaxed and endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide for 15 minutes. Antigen retrieval was achieved by microwaving the slides in citrate buffer (p.H. 6.0) for 18 minutes. The primary antibody AS (BD Transduction Laboratories, Oxford, United Kingdom) was applied for 60 minutes at 37°C (dilution 1:100), and staining was done with the avidin-biotin complex by using the DAKO streptavidin-avidin-biotin complex kit (DakoCytomation, Ely, United Kingdom). 3,3'-Diaminobenzidine was used as a chromogen (Vector Laboratories, Peterborough, United Kingdom), and the slides were then lightly counterstained with hematoxylin. Rat kidney sections were used AS-positive and AS-AS-negative controls. Two individuals (A.K. and M.S.) scored the slides independently and semiquantitatively, according to intensity of staining for AS protein: 0, + (weak); ++ (moderate); and +++ (strong). Difficult cases were reviewed together using a double-headed microscope to reach a consensus verdict.

Arginine depletion experiments. Cells were grown as described above, harvested using trypsin/versene, and replated using 1.5×10^5 per well of a six-well plate. Eighteen hours later, the medium was removed and cells were washed twice using endotoxin-free PBS. MPM cells were then cultured using 4 mL medium containing 2% dialyzed fetal bovine serum under different conditions of arginine and citrulline supplementation (-/+) as follows: arginine positive and citrulline positive (ARG⁺ CIT⁺; positive control); arginine negative

and citrulline positive (ARG⁻ CIT⁺); and without both amino acids (ARG⁻ CIT⁻; negative control). Cells were harvested at days 4 and 9, with cell counting done using a Vi-Cell Counter (Beckman Coulter, Fullerton, CA).

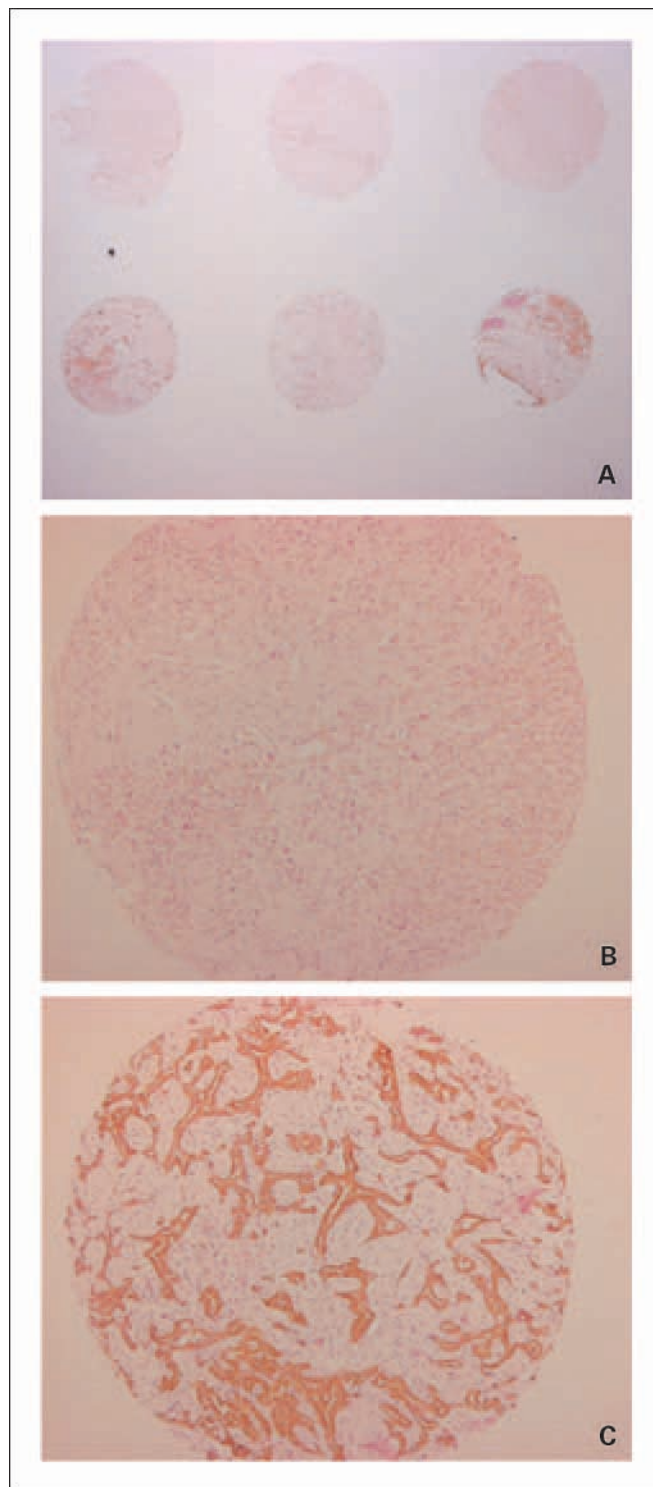


Fig. 3. Malignant mesothelioma tissue microarray. A, a representative low-power view (magnification, $\times 25$) of the tissue microarray after application of AS antibody: four of six cases are absent for AS protein. Medium-power view (magnification, $\times 100$) of an individual mesothelioma case negative for AS (B) and another positive for AS (C). Table 1 summarizes AS expression according to mesothelioma subtype.

Table 1. AS methylation status of MPM cell lines

AS mRNA levels	MPM cell line	AS methylation status
High	226, 2461	U
Intermediate	2052	UM
Very low	2591, MSTO	M

Abbreviations: U, unmethylated reaction; M, methylated reaction; UM, mixed PCR products.

Table 2. AS expression according to mesothelioma subtype

Tumor subtype (no. biopsies)	AS low expressors*	AS high expressors
Epithelioid (50)	28	22
Sarcomatoid (7)	7	0
Biphasic/mixed (25)	17	8
Total (82)	52 (63%)	30

*Intensity of AS staining: 0/+ (low expressor) and ++/+++ (high expressor).

BAX activation and mitochondrial depolarization. BAX activation was measured by exposure of the NH₂-terminal 6A7 epitope, which accompanies its unfolding as reported previously.⁷ Cells were harvested following incubation in complete or arginine-depleted medium as described previously. A two-stage immunocytochemistry used fixation and permeabilization using IntraStain (DAKO, Ely, United Kingdom) and labeling with primary mouse anti-human BAX 6A7 monoclonal antibody (Calbiochem, Nottingham, United Kingdom), and secondary anti-mouse Texas red-labeled antibody (Abcam, Cambridge, United Kingdom). Fluorescence was measured using a Coulter Epics XL flow cytometer using FL3 bandpass filter. Mitochondrial depolarization was measured by reduction in 3,3'-dihexyloxycarbocyanine, DiOC₆(3) (Molecular Probes, Eugene, OR). Cells were treated with 40 nmol/L DiOC₆(3) and incubated for 15 minutes at 37°C in the dark, and fluorescence was measured using the FL1 bandpass filter. Multiparameter analysis used counterstaining with 20 mg/mL propidium iodide (Sigma) to enable gating of dead cells. List mode data were analyzed offline using WinMDI2.8.⁸ The proportion of live cells with DiOC₆(3)_{low} PI_{low} was calculated and plotted.

Statistical analysis. InStat version 2.01 was used to test results for statistical significance (Bonferroni test).

Results

Reduced AS mRNA and absent AS protein expression in MPM cell lines. Seven MPM cell lines were analyzed for AS mRNA expression using quantitative real-time reverse transcription-PCR. Three (2591, 2052, and MSTO) of seven cell lines showed a marked reduction of AS mRNA comparable with the low levels expressed by a melanoma-negative control cell line and significantly lower than the positive ovarian cancer cell line (Fig. 1A). Next, we assessed AS protein expression by Western blotting. AS protein was strikingly absent in four (2591, 2052, MSTO, and 2452) of seven MPM cell lines (Fig. 1B).

9q34.11 locus of AS-negative MPM is intact: evidence for hypermethylation of the AS promoter. In view of the marked down-regulation of AS mRNA in three of the seven MPM cell lines, we did microarray-based CGH analysis. Molecular analysis by CGH array revealed no evidence of deletion in the 9q chromosome at the AS gene locus 9q34.11, in the three AS-negative nor AS-positive MPM cell lines (Fig. 2). Analysis of the AS locus revealed the presence of a CpG island in the 5' regulatory sequences of the gene, raising the possibility of epigenetic transcriptional silencing. To address this, we did methylation-specific PCR analysis in two cell lines abundantly expressing AS mRNA, two lacking detectable expression and

one with intermediate expression. There was complete methylation in the two cell lines lacking AS expression and partial methylation in the cell line with intermediate AS levels. There was no detectable methylation in the two cell lines abundantly expressing AS mRNA (Table 1). In summary, these results are consistent with methylation-dependent transcriptional silencing of the AS gene in a subset of MPM cell lines.

Absent AS expression in primary MPM. Next, we analyzed AS expression using a tissue microarray containing samples of primary MPM from patients treated at St. Bartholomew's Hospital. Fifty of 82 (63%) of tumors expressed markedly reduced or absent AS protein ('low expressors'), with the remaining samples showing moderate to strong levels of AS protein ('high expressors'; Fig. 3A-C; Table 2). Absence of AS expression was seen in the majority of MPM subtypes (i.e., epithelioid, sarcomatoid, and mixed tumors).

Arginine depletion abrogates cell growth in AS-negative tumors. Based on studies of arginine depletion in a variety of tumor cell lines, including the AS-negative melanoma and

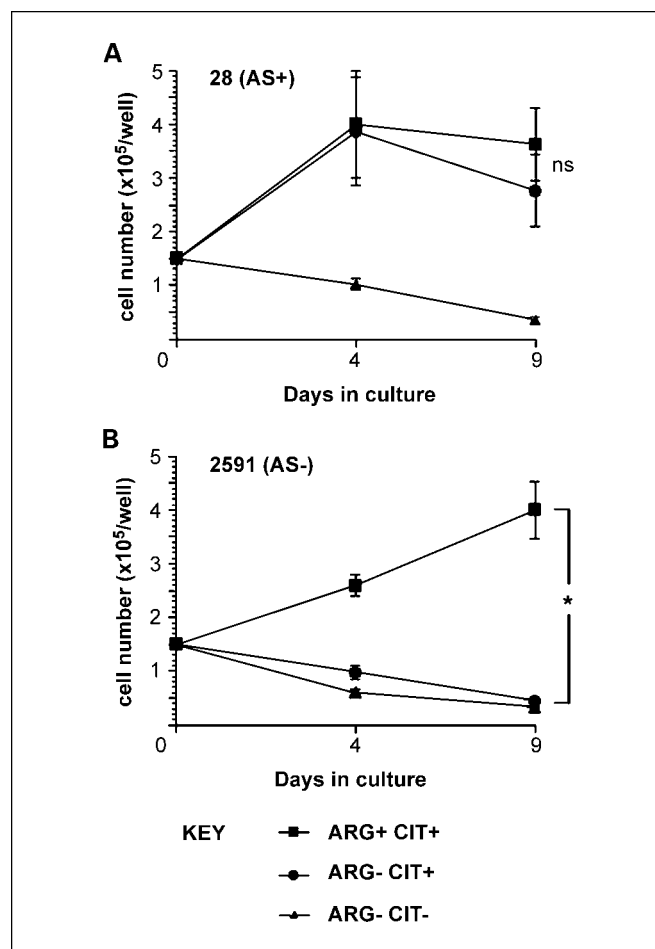


Fig. 4. The effect of arginine depletion on the growth of malignant mesothelioma cells. An AS-positive cell line (28) and two AS-negative cell lines (2591 and MSTO) were grown at a density of 1.5×10^5 per well (six-well plate). Three different medium conditions were used as described in Material and Methods: citrulline (CIT; +) and arginine (ARG; +); citrulline (+) and arginine (-); and citrulline (-) and arginine (-). Arginine depletion had (A) no effect on the growth of cell line 28 but induced (B) a marked decline in the growth of cell line 2591 by day 4 and persisting by day 9 of cell culture. The MSTO cell line yielded similar results to 2591 following arginine depletion (data not shown). Data are representative experiment done in triplicate and repeated thrice. ns, $P = 0.332$; *, $P < 0.0001$, Bonferroni test.

⁷ <http://www.jbc.org/cgi/content/full/273/17/10777>.

⁸ <http://facs.scripps.edu/software.html>.

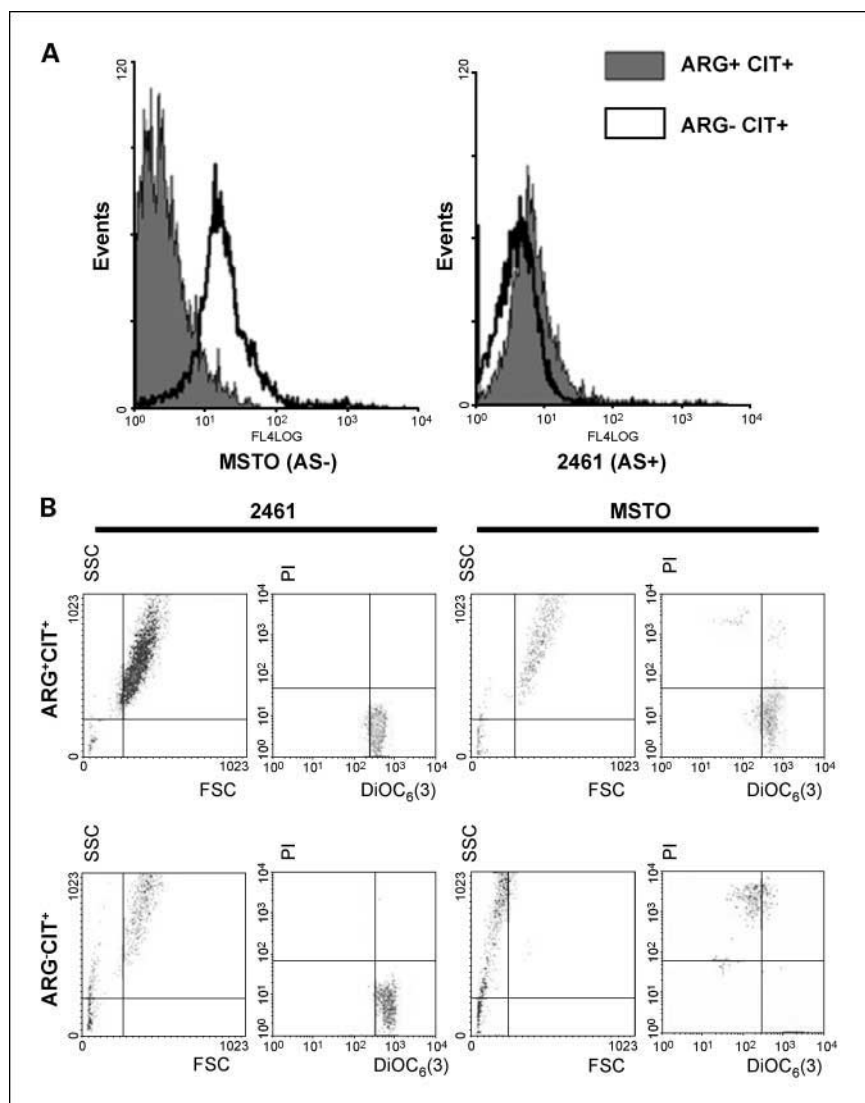


Fig. 5. Arginine depletion triggers apoptosis of AS-negative malignant mesothelioma cell lines. *A*, conformational activation of BAX measured by exposure of the 6A7 epitope is observed in AS-negative cell line MSTO but not the AS-positive cell line, 2461, following arginine depletion. *B*, 2461 mesothelioma cells with expression of AS remained viable in the absence of arginine as evidenced by no change in either forward scatter (FSC)/side scatter (SSC) or DiOC₆(3)/PI, which measures mitochondrial depolarization and plasma membrane integrity. In contrast, in the absence of arginine, MSTO cells lacking expression of AS exhibited both a marked reduction in forward scatter and mitochondrial membrane depolarization/loss of plasma membrane integrity consistent with cytotoxicity.

hepatocellular carcinoma (16–21), we tested the effects of arginine withdrawal in MPM. Supplementation of citrulline (a precursor molecule for AS) was required to distinguish between an essential and nonessential role for arginine in tumor metabolism (5). The AS-positive cell line 28 grew equally well in the presence and absence of arginine over a 9-day period (Fig. 4A). In contrast, the AS-negative cell line 2591 failed to grow in the absence of arginine with <90% of the initial cell number by day 9 (Fig. 4B). Similar results were obtained with a second AS-deficient cell line, MSTO (data not shown). As expected, absence of both citrulline and arginine in the culture medium induced marked loss of cell growth in both AS-positive and AS-negative cell lines.

AS-negative tumors undergo BAX activation and mitochondrial depolarization in the absence of arginine. To explore the processes underlying cytotoxicity of arginine depletion in AS-negative MPM, the conformational state of BAX was determined. In AS-negative MSTO, depletion of arginine led to markedly increased 6A7 fluorescence compared with AS-positive 2461 cells consistent with differential BAX conformation change and activation (Fig. 5A). Mitochondrial inner

membrane permeabilization associated with reduced DiOC₆(3) is an early consequence of BAX activation and occurred selectively in AS-negative MSTO cells but not AS-positive 2461 cells (Fig. 5B). In summary, AS-deficient tumors undergo cell death following withdrawal of arginine via a pathway involving the proapoptotic protein, BAX.

Discussion

This study confirms that the MPM cases we tested are predominantly AS negative and implicates arginine as an essential amino acid in MPM cell line growth. *In vitro* depletion of arginine resulted in marked apoptosis of tumor cells via up-regulation of BAX, as defined in one cell line, indicating that arginine deprivation therapy merits further investigation in patients with MPM.

In general, there was a good correlation between AS mRNA and protein levels in the MPM cell lines. We confirmed that the known locus of the AS gene on chromosome 9q was intact using the CGH array and that hypermethylation of the AS promoter site may explain the AS-negative status of some MPM

tumors. In contrast, the 2452 MPM cell line, with the clear disparity between abundant mRNA and absent AS protein, may reflect a less common post-transcriptional or translational block or increased proteosomal degradation. Consistent with the cell line data, however, the tissue microarray revealed an overall 63% 'low expressor' phenotype in the biopsies taken from patients with MPM. The tissue microarray represented the full spectrum of MPM histologic subtypes and enabled rapid characterization of AS expression in our patients.

Next, we confirmed that arginine was essential for the *in vitro* growth of AS-negative MPM cells. Depletion of this amino acid resulted in a major loss of tumor cells with apoptosis measurable within 48 hours of cell culture. Although not measured, peroxynitrite may account for the toxic effect of arginine withdrawal (22). Nevertheless, antiapoptotic molecules expressed in MPM, such as BCL-2, BCL-XL, and MCL-1, are bypassed, with activation of the BAX conformation.

Although arginine deprivation therapy as an anticancer strategy has been investigated for several decades (16–21), it is only recently that it has shown encouraging activity in patients with specific tumor types (23). The arginine-degrading drug, pegylated arginine deiminase (ADI-PEG 20) with its prolonged half-life, safety, and ability to suppress detectable levels of circulating arginine, has been assessed in patients with malignant melanoma and hepatocellular carcinoma, arginine auxotrophs on account of their AS negativity (16). Izzo et al. (24) reported a 47% (complete and partial) response rate in a phase I/II study of patients with advanced hepatocellular

carcinoma. More recently, Ascierto et al. (25) documented an overall 25% (complete and partial) response rate in patients with advanced metastatic melanoma treated with the same drug. Several patients with prolonged response were observed in both studies, indicating the need for further randomized studies. Based on these reports, ADI-PEG 20 received orphan drug designation in both the United States and, more recently, in Europe for the treatment of hepatocellular carcinoma.

The reason for the apparent loss of AS in tumors, such as melanoma, hepatocellular carcinoma, and MPM, remains unknown. There is evidence, however, that nitric oxide is critical to the survival of malignant melanoma (26), and it is possible that exogenous arginine may regulate this pathway far more effectively than arginine that is synthesized from tumor-derived AS. However, most tumors appear to express AS in abundance (27) and can rapidly up-regulate AS expression under conditions of low arginine (28). Further work is needed to address first the basis for differential AS expression between and within tumor types and second to explore the role that arginine plays in tumorigenesis. Moreover, arginine-catabolizing enzymes, such as ADI-PEG 20, may affect tumor growth via several pathways, including nitric oxide availability, polyamine handling, generation of tumor stroma, and tumor angiogenesis (29).

In summary, we have identified for the first time that MPM is auxotrophic for arginine; furthermore, withdrawal of arginine can induce apoptosis in a highly chemoresistant phenotype. A clinical trial is planned, testing the efficacy of arginine depletion in patients with AS-negative MPM.

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