Arginine Deiminase Resistance in Melanoma Cells Is Associated with Metabolic Reprogramming, Glucose Dependence and Glutamine Addiction

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Abstract

Many malignant human tumors, including melanomas are auxotrophic for arginine due to reduced expression of argininosuccinate synthetase1 (ASS1), the rate-limiting enzyme for arginine biosynthesis. Pegylated arginine deiminase (ADI-PEG20), which degrades extracellular arginine resulting in arginine deprivation, has shown favorable results in clinical trials for treating arginine-auxotrophic tumors. Drug resistance is the major obstacle for effective ADI-PEG20 usage. To elucidate mechanisms of resistance, we established several ADI-PEG20-resistant (ADI[R]) variants from A2058 and SK-Mel-2 melanoma cells. Compared with the parental lines, these ADI[R] variants showed the following characteristics: (i) all ADI[R] cell lines showed elevated ASS1 expression resulting from the constitutive binding of the transcription factor c-Myc on the *ASS1* promoter, suggesting that elevated ASS1 is the major mechanism of resistance; (ii) the ADI[R] cell lines exhibited enhanced AKT signaling and were preferentially sensitive to PI3K/AKT inhibitors, but reduced mTOR signaling and preferentially resistant to mTOR inhibitor; (iii) these variants showed enhanced expression of glucose transporter 1 and lactate dehydrogenase-A, reduced expression of pyruvate dehydrogenase, and elevated sensitivity to the glycolytic inhibitors, 2-deoxy-glucose and 3-bromopyruvate, consistent with the enhanced glycolytical pathway (the Warburg effect); (iv) the resistant cells showed higher glutamine dehydrogenase and glutaminase expression and were preferentially vulnerable to glutamine inhibitors. We demonstrated that c-Myc, not elevated ASS1 expression, is involved in upregulation of many of these enzymes because knockdown of c-Myc reduced their expression; whereas overexpressed ASS1 by

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transfection reduced their expression. This study identified multiple targets for overcoming ADI-PEG resistance in cancer chemotherapy using recombinant arginine-degrading enzymes.

Keywords

melanoma; argininosuccinate synthetase; Arginine Deiminase; glycolysis; glutamine metabolism

Introduction

The amino acid L-arginine has many physiological functions because it is a precursor for the biosyntheses of proteins, nitric oxide, polyamines, proline, glutamate, creatine and agmatine. It also plays important roles in immune response to antigen by impaired cytokine production and an arrest in T cell proliferation (1, 2). Arginine is primarily synthesized by 2 sequential enzymatic reactions in the urea cycle: argininosuccinate synthetase -1 (ASS1), which catalyzes the reaction of L-citrulline and aspartic acid to argininosuccinate, and argininosuccinate lyase (ASL) which recycles argininosuccinate back to arginine with the production of fumaric acid, an intermediate in the tricarboxylic acid (TCA) cycle (Fig. 1).

Many advanced human tumors, including melanomas, renal cell carcinomas, hepatocellular carcinomas, mesotheliomas, prostate cancers, and small cell lung cancers do not express ASS1 (3). ASS1-negative tumor cells therefore require arginine from the circulation for survival, whereas normal cells that synthesize arginine endogenously will survive under arginine deprivation conditions. Arginine deiminase (ADI) (4–7) and human arginase I (4, 8, 9) two arginine-depleting recombinant proteins (Fig. 1), have been under clinical development for treating arginine-auxotrophic tumors. ADI is produced by *Mycoplasma* spp. and humans do not produce this enzyme. Like most recombinant bacterial proteins, it is immunogenic and has a short half-life *in vivo*. Pegylated ADI (ADI-PEG20) has been formulated for clinical use. Clinical trials using ADI-PEG20 for treating various cancers including melanoma have produced favorable results (5, 6).

Drug resistance remains an important contributor for the treatment failure, owing to re-expression of the once-silenced ASS1. Using a cultured melanoma cell system, we previously demonstrated that transcriptional regulation of ASS1 by ADI-PEG20 involves a switch of promoter binding: c-Myc which functions as a positive regulator replaces hypoxia-inducible factor HIF-1 which functions as a negative regulator (10, 11).

The current investigation was initiated to elucidate resistance mechanisms of ADI-PEG20 in melanoma cells. We developed several stable ADI-resistant (ADI[R]) cell lines and found that compared with their parental counterparts, these ADI[R] variants exhibited elevated glycolytic metabolic activities and were preferentially sensitive to the killing by glycolytic inhibitors. Moreover, these ADI[R] cells also exhibited elevated expression of the kidney-type glutaminase (GLS1) and glutamine dehydrogenase (GDH), key enzymes for glutaminolysis, and were preferentially sensitive to glutamine inhibitors. These results demonstrate that ADI resistance in arginine-auxotrophic melanoma cells is associated with metabolic reprogramming that provides potential targets for overcoming the resistance.

Materials and Methods

Reagents, Antibodies and shRNA

Reagents were obtained from the following sources: ADI-PEG20 (specific activity, 5–10 IU/mg) from Polaris Pharmaceuticals, Inc. (San Diego, CA); LY294002, 2-deoxy-D-glucose (2-DG), 3-bromopyruvate (3-BP), 6-diazo-5-oxo-L-norleucine (DON), azaserine, deferoxamine

mesylate (DFX), and CoCl$_2$, from Sigma-Aldridge (St. Louis, MO); perifosine from Cayman Chemical (Ann Arbor, MI); temsirolimus (CCI-779) from Selleckchem (Houston, TX). Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was prepared according to a previous report (12).

Antibodies were obtained from the following sources: mouse anti-human ASS1 monoclonal antibody from Polaris Pharmaceuticals; rabbit anti-argininosuccinate lyase (ASL) and rabbit anti-c-Myc (N262), rabbit anti-MCT1 and MCT4 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-glucose transporter 1 (Glut-1) from FabGennix (Frisco, TX); antibodies against lactate dehydrogenase A (LDH-A), pyruvate dehydrogenase (PDH), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), mTORC1, p70S6 Kinase, p-p70S6 Kinase (Thr389), 4E-BP, eIF2 , p-eIF2 (Ser51), ASCT2 (SLC15), AMPK , and p-AMPK (Thr172) from Cell Signaling (Danvers, MA); rabbit anti-kidney type glutaminase (GLS1) and rabbit anti-glutamine dehydrogenase (GDH) from Abcam (Cambridge, MA); and rabbit anti- -tubulin antibodies from Sigma-Aldrich (St Louis, MO).

Recombinant shRNA for c-Myc were purchased from Sigma-Aldridge.

Cell culture and establishment of ADI^R Cells

A2058, SK-Mel-2, and A375 melanoma cells were purchased from American Type Culture Collection Center without further authentication. All cell cultures were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 5% CO$_2$ atmosphere.

ADI^R cell lines were established by selecting the surviving population from A2058 and SK-Mel-2 cells that were continuously exposed to ADI-PEG20 with stepwise increases in concentrations, starting from 0.1 μg/mL and 0.05 μg/mL for A2058 and SK-Mel-2, respectively. Independent ADI^R cell lines were established from single cells using 96-well-plate. Increased resistance in these clones were further developed by increased drug concentrations with 0.025 μg/mL increments for over 6 months, at which time the resistant cells were able to survive at 0.9 μg/mL of ADI-PEG20. Cells were routinely maintained at 0.3 μg/mL of ADI-PEG20.

Glucose uptake

Glucose transport was measured according to the procedure described (13). In brief, the experiments were started by adding 1 μCi of 2-deoxy-[H^3]-D-glucose (2-DG) (5–10 Ci/mmol) into exponentially growing A2058 cells (5×10^5 in 6-wall, Corning). Cultured cells were maintained at 37°C for 20 min. Cells were harvested, washed 3 times with phosphate-buffered saline, and solubilized in 0.1N NaOH and the radioactivity was measured in a liquid scintillation counter.

Establishment of stable ASS1-overexpressing cell lines

The plasmid pCMV6-ASS1 carrying human full-length *ASS1* cDNA sequence (GenBank, NM_000050) (Origene, Rockville, MD) and empty vector (pCMV6) were transfected into A2058 and A375 cells using lipofectamine and positive clones were selected with G418.

Other procedures

Western blotting, chromatin immunoprecipitation (ChIP), cytotoxicity assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were performed as described previously (10, 11).

Statistical Analyses

The IC$_{50}$ values were obtained from nonlinear regression analysis of concentration-effect curves by the GraphPad Prism Software and represented by mean ± standard deviation of three independent experiments. For analysis with only two-groups (parental and ADI^R cells), Student's *t* test was used. The IC$_{50}$ values were plotted in reference to that of A2058 which was set at 1.0. Significance was determined when $p < 0.05$.

Results

Elevated ASS1 expression in ADI^R cell lines

We established 5 independent ADI^R variants from A2058 (A2058-R1 to A2058-R5) and 4 SK-Mel-2 (SK-Mel-2-R1 to SK-Mel-2-R4) lines. Cytotoxicity tests showed that all these cell lines were more resistant to ADI-PEG20 than were their respective parental cell lines. More than 95% of all ADI^R-A2058 cells could survive at 0.9 μg/mL ADI-PEG20 for 48 hr treatment, whereas < 25% of the parental cells survived (Fig. 2A). Likewise, treating SK-Mel-2 cells with 0.9 μg/ml ADI-PEG20 almost completely killed the cells, whereas >70% of ADI^R SK-Mel-2 cells were able to survive under the same treatment (Fig. 2B).

All the ADI^R-A2058 cells exhibited increased ASS1 expression (about 15-fold by densitometry, not shown) as compared with that in the drug-sensitive counterpart (Fig. 2C). Likewise, all the ADI^R-SK-Mel-2 cells had higher ASS1 expression levels than did the parental cell lines, but the SK-Mel-2-R1 and SK-Mel-2-R2 cell lines had lower ASS1 levels than did the SK-Mel-2-R3 and SK-Mel-2-R4 lines (Fig. 2D). Reduced expression of ASS1 in the SK-Mel-2-R1 and SK-Mel-2-R2 cell lines was correlated with less resistance to ADI-PEG20 (Fig. 2B). These observations, together with our previous finding that modulation of ASS1 levels affects cell sensitivity to ADI-PEG20 (4, 10), strongly support that ASS1 expression is the predominant mechanism for acquired resistance to ADI-PEG20 in cultured melanoma cells.

Roles of c-Myc and HIF-1α in the regulation of ASS1 expression in ADI^R cell lines

We previously demonstrated that induction of ASS1 expression by ADI-PEG20 in melanoma cells was associated with the up-regulation of c-Myc which functions as a positive regulator, and the down-regulation of HIF-1 which functions as a negative regulator (10, 11). Figs. 2C and 2D show that c-Myc expression levels were similar between all ADI^R variants and their respective parental cell lines; whereas reduced HIF-1 expression levels were seen in A2058-R2, A2058-R4, A2058-R5, and all ADI^R-SK-Mel-2 lines, but not in A2058-R1 and A2058-R3 cells. The observations that no elevated expression of c-Myc and no reduction of HIF-1 expression in all ADI^R cells prompted us to investigate whether c-Myc and HIF-1 remain important for regulating ASS1 expression in these ADI^R cell lines.

We chose 4 representative ADI^R cell lines (A2058-R1 which expressed higher HIF-1 levels than the control, and A2058-R2, SK-Mel-2-R1 and SK-Mel-2-R3 which did not express detectable levels of HIF-1). To investigate whether accumulation of HIF-1 would suppress ASS1 expression in the ADI^R cell lines, we treated these cells and their drug sensitive cells (as control) with hypoxic mimics, DFX and CoCl$_2$ (14). Treating A2058 or SK-Mel-2 cells with DFX or CoCl$_2$ suppressed ADI-PEG20-induced ASS1 expression (Fig. 3A, left). However, no suppression of ASS1 expression was found in A2058-R1, A2058-R2 and SK-Mel-2-R3, except SK-Mel-2-R1 cells (Fig. 3A, right). The suppression of ASS1 expression in SK-Mel-2-R1 cells by elevated HIF-1, but not in the other ADI^R cell lines may have been due to the lower expression levels of ASS1 in SK-Mel-2-R1 cells as compared with those in the other cell lines (Fig. 2D). These results suggest that the extent of

ASS1 suppression by over-expressed HIF-1α may depend upon the intrinsic ASS1 expression levels.

To investigate the role of c-Myc in regulating *ASS1* expression in ADI^R variants, we used c-Myc shRNA strategy. Knockdown of c-Myc expression by 2 independent shRNA in lentiviral vectors downregulated ASS1 expression (Fig. 3B), supporting the positive role of c-Myc in the regulation of ASS1 in the ADI^R cells.

We also performed ChIP assay to determine the occupancy of HIF-1α and c-Myc at the *ASS*1 promoter. Consistent with our previous results (10), we found that HIF-1α, but not c-Myc, binds to the *ASS*1 promoter in the parental A2058 (Fig. 3C, lane 1) and SK-Mel-2 cells (Fig. 3D, lane 1). In contrast, in all the ADI^R variants investigated, c-Myc, not HIF-1α, binds to the *ASS1* promoter (Figs. 3C & 3D, lanes 2 & 3). These results taken together, demonstrate that HIF-1α and c-Myc remain important in transcriptional regulation of ASS1 expression in ADI^R variants.

ADI^R cell lines are sensitive to PI3K/AKT inhibitors

We previously observed that A2058 and SK-Mel-2 cells treated with ADI-PEG20 activated the Ras/PI3K/AKT-1 signaling cascade, resulting in c-Myc stabilization for ASS1 upregulation (11). Moreover, several recent reports suggested that PI3K/AKT play important roles in regulating c-Myc expression (15–17). Activation of PI3K/AKT-1 signal is elicited by phosphorylation of AKT-1 at threonine 308. We performed Western blotting analyses of 5 ADI^R-A2058 cell lines using anti-phosphorylated AKT antibody and found that all these cells contained activated AKT-1 signal (Fig. 4A). Activation of AKT-1 in these ADI^R cells was reflected by increased sensitivities to the killing by LY294002 and perifosine which inhibit PI3K and AKT1 activities, respectively (Fig. 4B).

Downregulated mTOR signaling in ADI^R Cell Lines

Three well-characterized sensing mechanisms for cellular amino acid and/or nutritional deprivation in eukaryotic cells include the mammalian target of rapamycin complex 1 (mTORC1), general amino acid control nonderepressible 2 (GCN2) and AMP-dependent protein kinase (AMPK) (18, 19). mTORC1 phosphorylates and activates protein kinase p70S6 kinase 1 (S6K1) which subsequently activates mRNA cap binding by phosphorylating eIF-4B for the formation of translational initiation complex. GCN2 is the only kinase for eukaryotic initiation factor 2α (eIF2α) that senses the absence of one or more amino acids by virtue of direct binding to uncharged cognate tRNA (20). Phosphorylation of eIF2α (p-eIF2α) by activated GCN2 prevents the assembly of eIF2-GTP-Met-tRNA^{Met} translation initiation complex, resulting in reduction of general protein synthesis (21, 22). AMPK is a metabolic sensor of energy crisis resulting from nutritional deprivation (23). We investigated the expression of these signals in 5 ADI^R-A2058 cells. Fig. 4C shows that expression levels of mTOR and p-mTOR were drastically reduced in all 5 ADIR cell lines. Likewise, the downstream signal for mTOR, p70S6K was also downregulated. Downregulation of these signals in these ADI^R cells is reflected by virtue of their resistance to clinically approved mTOR inhibitor, temsirolimus (CCI-779) (24) (Fig. 4D). Expression levels of AMPKα are variable among the 5 ADI^R cell lines (Fig. 4C) and levels of phosphorylated AMPKα were too low to be detected in all cells (not shown). No apparent difference in eIF2α levels was observed between ADI^R-A2058 cells and their parental cells, but levels of phosphorylated eIF2α (p-eIF2α) were low in all the cell lines investigated, using cell lysate prepared from A2058 cells treated with ADI-PEG20 for 24 hr as a positive control (Fig. 4C). These results demonstrated that mTOR is the only signal that is consistently downregulated in the ADI^R cells.

ADI[R] cells are associated with enhanced expression of ASL and glycolytic activities (the Warburg effect)

We then investigated whether ADI-PEG20 resistance would alter the metabolic programs. Because ASL is directly downstream from ASS1 in the urea cycle (Fig. 1), we performed Western blotting of its expression in 5 ADI[R]-A2058 lines. We found that ASL expression levels were elevated in all 5 ADI[R] cell lines (Fig. 5A).

Next, we investigated the expression of several key players involved in the glycolytic pathway in the ADI[R] cell lines. We first investigated Glut1 which is the major transporter for glucose and found that all 5 ADI[R]-A2058 cells showed about 8-fold higher Glut1 levels than those in A2058 parental cells. Elevated Glut1 expression in these the ADI[R] cell lines was correlated with the enhanced transport of [^{3}H]-2-DG, a non-metabolizable form of glucose (Fig. 5B). Compared with the parental cell lines, the ADI[R] variants showed higher levels of LDH-A which catalyzes the conversion of pyruvate into lactate (Fig. 1), but lower levels of PDH (Fig. 5A). PDH is the key enzyme that catalyzes the conversion of pyruvate into acetyl CoA, an important metabolic intermediate in the TCA cycle (Fig. 1).

Monocarboxylate transporter family (MCT, SLC16) catalyzes the rapid transport across the plasma membrane of many monocarboxylates including lactate (25). Since these ADIR cells show elevated expression of LDH-A, we examined the expression levels of MCT1 and MCT4 in ADI[R] A2058 cells. Results showed that levels of MCT1 were increased in A2058-R1, A2058-R2, A2058-R4, and A2058-R5, but reduced in A2058-R3; whereas levels of MCT4 were increased in A2058-R2, A2058-R3, and A2058-R5, but reduced in A2058-R4, and no change in A2058-R1 (data not shown). Thus, the expression levels of MCT1 and MCT4 are heterogeneous among the 5 ADI[R]-A2058 cells. Their roles in ADI resistance are not certain.

ADI[R] cell lines are preferentially sensitive to glycolytic inhibitors

Since ADI[R] cells exhibit elevated expression of Glut1 and enhanced glucose uptake, known as the Warburg effect, we asked whether these cells would be preferentially killed by 2-DG, a commonly used inhibitor of glycolysis (26–28). We found that, indeed, all the 5 ADI[R]-A2058 variants exhibited enhanced sensitivity to 2-DG (Fig. 5C). Moreover, we found that these 5 ADI[R] cells were also vulnerable to another glucolytic inhibitor 3-BP, a lactate/pyruvate analogue (29, 30) (Fig. 5C), demonstrating that these ADI[R] cells are preferentially sensitive to glycolytic inhibitors as compared with their parental cell lines.

ADI[R] cells exhibit elevated glutaminolytic metabolism and increased sensitivity to glutaminase Inhibitors

Two key enzymes in glutaminolytic metabolism, glutaminase (GLS) and glutamine dehydrogenase (GDH), catalyze the 2-step sequential conversion of glutamine into -ketoglutarate via glutamate intermediate (Fig. 1). Mammals express 2 GLS isoforms, kidney-type (or GLS1) and liver-type (GLS2). GLS1 is expressed in a broad spectrum of cell lines. We demonstrated elevated expression of GLS1 and GDH in all 5 ADI[R]-A2058 cell lines as compared with those in the parental control, indicating the increased glutaminolytic activities in these ADI[R] variants (Fig. 5D). These results were supported by the demonstration that all the 5 ADI[R] cell lines were associated by preferential sensitivity to GLS inhibitors, DON, azaserine, and BPTES (Fig. 5E). Despite increased glutaminolytic enzymes in these ADI[R] cell lines, we found only A2058-R3 exhibited elevated ASCT2 glutamine transporter among the 5 ADI[R] A2058 cells (data not shown).

C-Myc, but not elevated ASS1, contributes to the enhanced glycolytic and glutaminolytic metabolisms in ADI[R] cell lines

To investigate the kinetics of induction of these glycolytic and glutaminolytic enzymes by ADI-PEG20 in the ADI[R] cells, we performed an ADI-PEG20 time-course treatment of A2058 cells from 8 to 72 hrs. We found that ADI-PEG20 induced not only ASS1 and c-Myc, but also Glut1, LDH-A, GLS1 (but not GDH) within this time frame. These results indicate that induction of ASS1, Glut1, LDH-A, occurred very early during the development of ADI resistance, and elevation of GDH levels occurred subsequently (Fig. 6A).

Since elevated expression of ASS1 is the principal mechanism of ADI resistance, we investigated whether enhanced expression of these enzymes was the consequence of ASS1 upregulation in the ADI[R] cell lines. To this end, we used transfection to establish 2 independently overexpressed ASS1 cell lines in A2058 and A375 cells. While no apparent alteration of HIF-1 levels was discernible even using prolonged exposure of the Western blots (not shown). We found that expression of c-Myc, Glut1, LDH-A, GLS1 and GDH was reduced (Fig. 6B). This expression profile is directly opposite from that in the ADI[R] cell lines. These results demonstrate that elevated expression of these enzymes in ADI[R] cells was not likely due to the enhanced ASS1 expression.

We next investigated whether c-Myc plays a role in regulating the glycolytic and glutaminolytic enzymes in these ADI[R] cells, in light that c-Myc has been recognized as an important transcription regulator of 10% to 20% of total cellular genes through E-box interactions in mammalian cells (31, 32). A2058-R1 and A2058-R2 cells were treated with 2 independent c-Myc shRNA for 48 hr. Although the shRNA we used could only partially reduce c-Myc levels, we found reduced expression of Glut1 and GLS1 in these ADI[R] cells as compared with those in the scramble shRNA-treated controls, whereas levels of LDH-A and GDH were not affected. The inability of reducing LDH-A and GDH suggest that either other mechanisms are involved for the regulation of LDH-A and GDH or regulation of these enzymes by c-Myc is more stringent than Glut1 or GLS1. In any event, these results support that c-Myc plays a role in regulating metabolic rewiring during the development of ADI-PEG20 resistance.

Expression of enzymes involved in the lipid biosynthesis in the ADI[R] cell lines

We also determined the expression of enzymes involved in fatty acid biosynthesis in ADI[R] cell lines. Compared with their parental counterpart, all the ADI[R] A2058 cell lines, except A2058-R4, had lower expression levels of ACC which catalyzes the conversion of acetyl-CoA into malonyl-CoA (Fig. 1) (data not shown), and all the ADI[R] A2058 cell lines, except A2058-R2, had lower levels of FAS which converts malonyl-CoA into palmitoyl-CoA (data not shown). These results suggest that fatty acid metabolism may also be reduced in the ADI[R] cell lines compared with their parental counterpart.

Discussion

The discovery that many human malignant tumors do not express ASS1 and are arginine-auxotrophic provides a mechanistic basis for using recombinant arginine-depleting enzymes in targeted therapy of these diseases (4). In this communication, we demonstrate that in a panel of 9 independently established ADI[R] cell lines all expressed elevated levels of ASS1, further strengthening the role of ASS1 in ADI resistance. These ADI[R] cell lines serve as a valuable resource for investigating the metabolic reprogramming associated with ADI resistance as summarized in Fig 1. Several important findings are discussed below.

We found that these ADI[R] variants exhibited elevated AKT signaling and are preferentially sensitive to PI3K/AKT inhibitors. These findings support the important role of this pathway

in ADI-PEG20 resistance (11). We also observed that expression of mTOR and its downstream signal p70S6K was downregulated in all ADI[R] cells. While the canonical pathway of mTOR regulation is through the PI3K/AKT pathway (33), however, downregulation of mTOR independent of this pathway has also been reported (34). The molecular mechanism for downregulation of mTOR signal in ADI resistance remains to be investigated.

We further demonstrated that ASL was elevated in these ADI[R] lines, suggesting that the ADI[R] cells may require robust ASS1/ASL activities in the urea cycle to overcome ADI-PEG20-induced autophagy and apoptosis in the otherwise arginine-auxotrophic cancer cells (11, 35). Although ASS1 has often been considered as the rate-limiting enzyme for the biosynthesis of arginine (36), our results suggest that ADI[R] cells may require additional enzymes to replenish arginine under prolong arginine deprivation conditions. Our results are consistent with recent findings that several enzymes in the urea cycle may be co-regulated (37), including ASS1, ASL, nitric oxide synthetase, and cationic amino acid transporter 1 and may form a supermolecular complex to perform important physiological function (38).

We found that these ADI[R] cells have altered metabolic program toward aerobic glycolysis by enhancing Glut1 and LDH-A expression but reducing PDH expression, exhibiting the Warburg phenomenon. Since Warburg's work in 1929, it has been evidenced that cancer cells, even under aerobic conditions, frequently upregulate glucose metabolism leading to a high uptake and use of glucose, but a moderate rate of mitochondrial respiration (39, 40). This characteristic has led to the development of 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) in positron emission tomography (PET) for the imaging of primary and metastatic tumors in patients (41). Our observations that ADI[R] cells exhibited elevated Warburg phenomenon suggest that it may be possible to use FDG-PET for monitoring the development of ADI resistance during cancer chemotherapy. In clinical setting, melanoma is known to be strongly PET positive and usually become less FDG avid when tumor response to treatment. Once tumors become resistant to ADI-PEG20, melanoma will again become FDG avid. This was also observed clinically when patient failed ADI-PEG20.

Metabolic reprogramming in ADI[R] cell lines also include elevated expression of GLS1 and GDH, another common phenomenon in cancer cells (42, 43). These enzymes are important for producing -ketoglutarate, an important metabolite in the TCA cycle. Tumor cells fuel their metabolism with glucose and glutamine as important bioenergetic sources for cell proliferation. The dependence of these ADI[R] cells to glucose and glutamine for growth was demonstrated by the findings that these ADI[R] cells exhibited elevated sensitivity to the killing by glucose (2-DG and 3-BP) and glutamine inhibitors (DON, azaserine, and BPTES). While DON and azaserine have long been known to be toxic in human clinical evaluations (44), presumably due to the presence of chemically reactive diazo group. The increased sensitivity of these ADI[R] cells to BPTES demonstrated in this study may have promising therapeutic implication. New generation of GLS1 inhibitors such as BPTES derivatives which are devoid of diazo group found in DON and azaserine (12), as well as compound 968 and its derivatives, another structurally distinct series of GLS1 inhibitor (45, 46) that are currently under clinical evaluations, may pave the way for future clinical applications.

We also found that c-Myc plays important roles in the metabolic rewiring of ADI resistance. Previous studies have demonstrated that c-Myc regulates genes involved in the glycolytic pathway by directly binding to the E-box located at the promoters of these genes (47). C-Myc can also upregulates glutamine metabolism via transcriptional repression of miR-23a and miR-23b (48). Although levels of c-Myc were not elevated in these ADI[R] cells, however, our kinetic analysis demonstrated that expression of c-Myc was induced by ADI-PEG20 within the same time-frame when many of these enzymes were also induced.

Elevated levels of glycolytic and glutaminolytic pathways apparently are needed for the maintenance of ADI resistance, because inhibitors to these pathways overcome the resistance. The observation that no elevated c-Myc levels in all the 9 ADI[R] cell lines, despite their early induction, suggests that persistently high-levels of c-Myc may be disadvantageous for cell growth during the development of ADI resistance.

We also discovered that elevated expression of ASS1 by transfection downregulates Glut1, LDH-A, GLS1, and GDH expression, demonstrating that modulating ASS1 levels can influence the expression of other enzymes in distance, i.e. glycolytic and glutaminolytic pathways. While the precise mechanism(s) remain to be investigated, downregulation of c-Myc may be responsible for the reduced expression of these enzymes in these *ASS*1-treansfected cells, although this remain to be elucidated. Moreover, the findings of reduced levels c-Myc levels in these ASS1-transfected cells may explain why ADI[R] cells did not show elevated c-Myc, as this may be due to feedback inhibition mechanism of c-Myc regulation once ASS1 is overly expression. These possible mechanisms would further support the important roles of c-Myc in the regulation of glycolytic and glutaminolytic metabolism in melanoma cells.

Finally, the identification of metabolic reprogramming associated with ADI resistance in the arginine-auxotrophic melanoma model bears important clinical implications for targeted therapy of arginine-auxotrophic tumors. Many anti-tumor small molecules targeting PI3K/AKT, glycolytic and glutaminolytic signalings have been approved for clinical use. Combination of these agents with arginine-degrading enzymes, such as ADI-PEG20 or pegylated human arginase I may improve the treatment efficacy of many human cancers that require arginine for survival.

Acknowledgments

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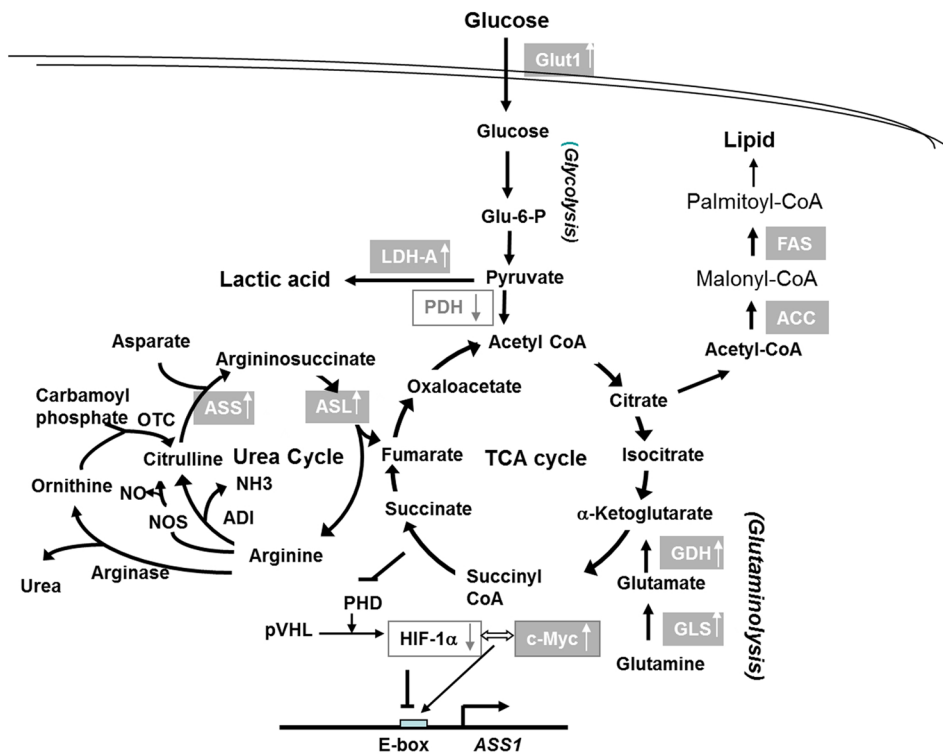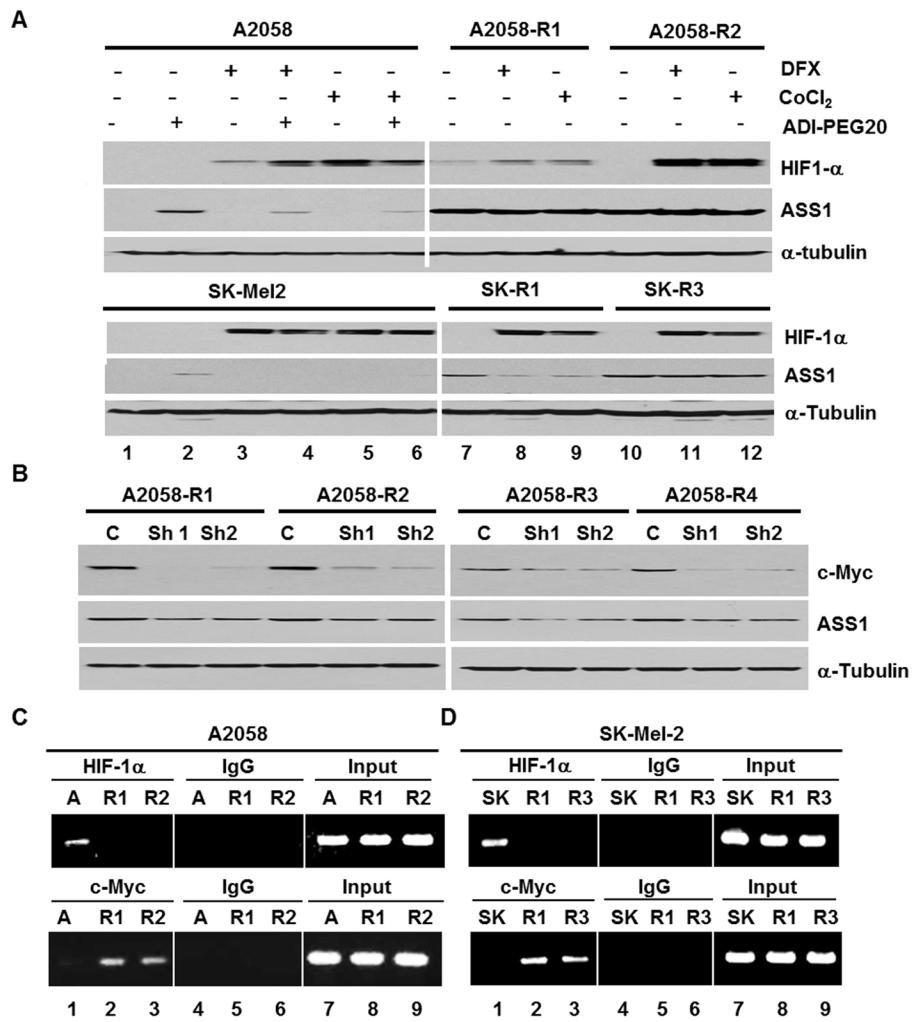
Figure 1.
The metabolic interrelationships of glycolysis, the TCA cycle, the urea cycle, and glutaminolysis. Upward arrows in the boxes denote that the expression is increased in the ADI[R] cell lines or can be induced by ADI-PEG20; downward arrows in the boxes denote downregulation. Abbreviations are: ACC=acetyl CoA carboxylase, ADI=arginine deiminase, ASL=argininosuccinate lyase, ASS1=argininosuccinate synthetase 1, FAS=fatty acid synthase, Glu-6-P=glucose-6-phosphate; GDH=glutamine dehydrogenase, Glut1=glucose transporter 1, GLS1=kidney-type glutaminase, LDH-A=lactate dehydrogenase-A, NOS=nitroxide synthetase, OTC=ornithine carbamoyltransferase, PDH=pyruvate dehydrogenase, PHD=prolyl hydroxylation, TCA= tricaboxylic acid cycle,
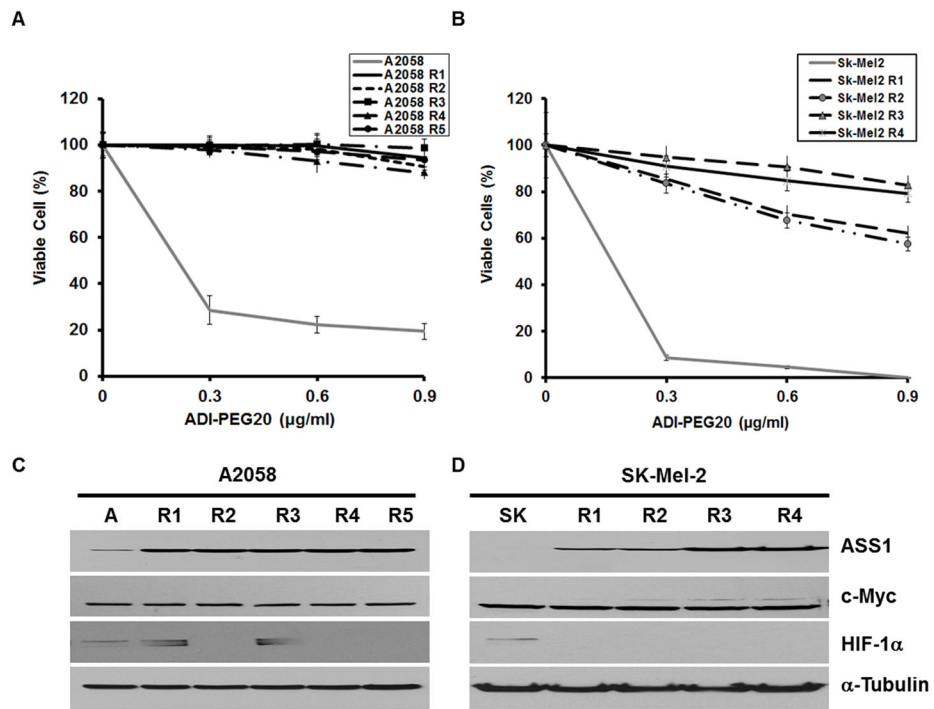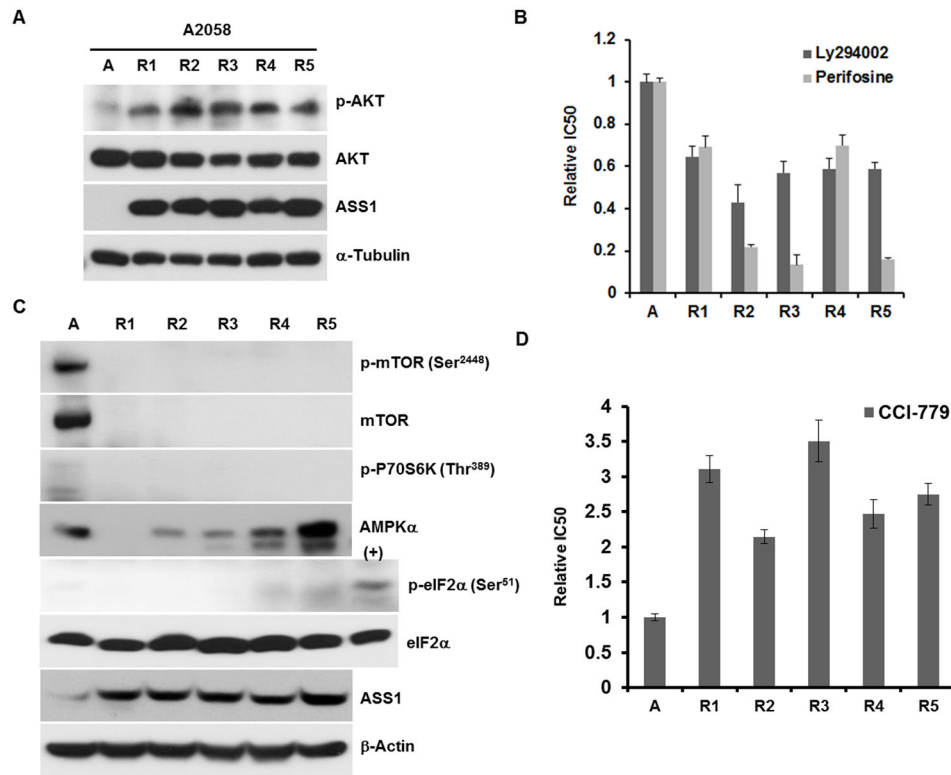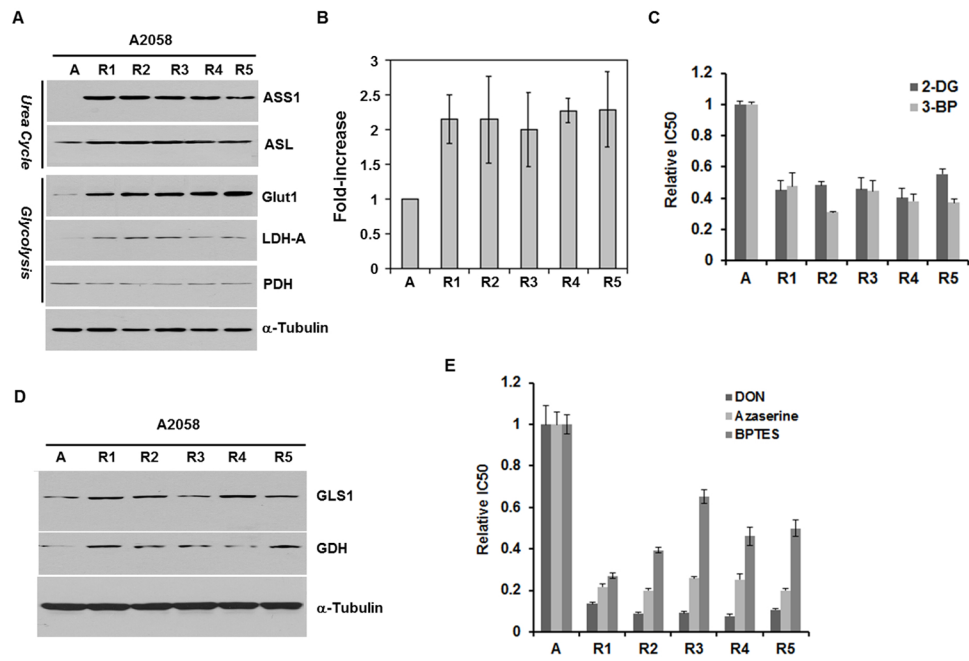
Figure 2.
Survivability test and protein expression of ADI^R variants. A, A2058 and its ADI^R variants
(A2058-R1 to A2058-R5) and B, SK-Mel-2 and its ADI^R variants (SK-R1 to SK-R4) were
treated with different concentrations of ADI-PEG20 for 3 days. Viability of the treated cells
was determined by MTT assay. C, Western blotting analyses of ASS1, c-Myc, HIF-1 , and
 -tubulin expression in A2058 (denoted by A) and its ADI^R variants (R1 to R5). D, Western
blotting of protein expression in SK-Mel-2 (SK) and its ADI^R lines (R1 to R4).

Figure 3.
Roles of c-Myc and HIF-1 in the expression of ASS1 in ADI[R] cell lines and their parental cell lines. A, Effects of HIF-1 accumulation by hypoxia mimics, DFX (50 μM) or CoCl₂, (100 μM) on the expression of ASS1 in A2058, SK-Mel2 induced by ADI-P EG 20 (0.3 μg/ mL) and their ADI[R] cell lines. Note that accumulation of HIF-1 suppresses ASS1 expression in SK-R1 but not in SK-R3. B, knockdown of c-Myc by shRNA (Sh1 and Sh2) suppressed ASS1 expression in the indicated ADI[R] cell lines. C, ChIP assay of HIF-1 and c-Myc interactions with the *ASS1* promoter in A2058 (denote by A, lane 1), A2058-R1 (lane 2) and A2058-R2 (lane 3). *Top,* the antibodies used for ChIP and control *IgG. Input,* genomic DNA prior to immunoprecipitation. D, similar experiment was performed in SK-Mel-2 (SK) and its ADI[R] cell lines (R1 and R3). *ASS1* promoter sequences from ChIP were quantified by PCR assay.
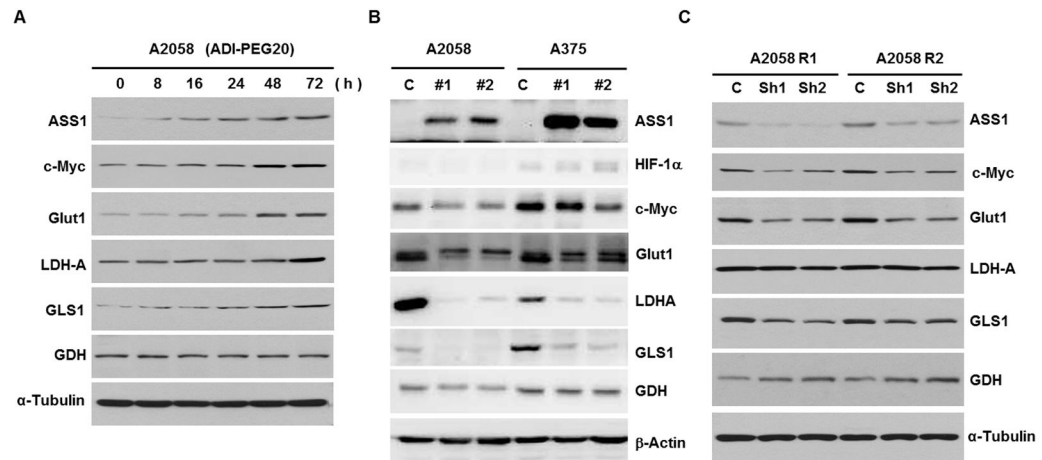
Figure 4.
Analyses of protein expression in A2058 and its 5 ADI^R cells. A, activated phosphorylation AKT (p-AKT) signaling; B, enhanced sensitivity of ADI^R cells to the PI3K inhibitor (LY294002) and AKT (perifosine) as compared with their parental line. C, Western blotting analyses of the mTOR signaling, AMPK , and eIF1 . D, Sensitivity of A2058 and its ADI^R cells to mTOR inhibitor CCI-779. Cells were incubated with CCI-779 at concentrations ranging from 1 to 100 μM for 72 h and their viabilities were determined by MTT assay.

Figure 5.
ADI[R] cell lines exhibited elevated glycolytic and glutaminolytic metabolism and increased sensitivity to glucose and glutaminase inhibitors A, Western blots of glucolytic enzymes in A2058 and its ADI[R] cells; B, enhanced uptake of 2-DG in ADI[R] cell lines as compared with A2058 cells; C, enhanced sensitivity of ADI[R] cell lines to glucolysis inhibitors 2-DG and 3-BP as compared with A2058 cells; D, expression levels of GLS1 and GDH in A2058 and its ADI[R] cell lines. E, enhanced sensitivity of ADI[R] cells to glutamine inhibitors, DON, azerserine and BPTES, as compared with A2058 cells.

Figure 6.
Western blotting analyses of protein expression. A, time-course induction of various enzymes by ADI-PEG20 treatment (0.3 μg/mL) in A2058 cells; B, effects of ASS1-overexpression in A2058 and A375 cell lines by *ASS1* transfection (2 independent cell lines each) on the expression of several proteins as indicated. C, effects of c-Myc knockdown on the expression of proteins as indicated in two ADI[R]-A2058 cell lines.