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Targeting glutamine metabolism sensitizes melanoma cells to TRAIL-induced death

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ABSTRACT

Targeting specific metabolic pathways has emerged for cancer therapeutics. For melanoma, metabolic studies have solely focused on high glucose uptake. By contrast, little is known regarding addiction to glutamine. Using five melanoma lines and two normal cell types, addition of aminooxyacetate (AOA), an inhibitor of glutamate-dependent transaminase regulating glutaminolytic pathway, two lines underwent low levels of apoptosis (>30%), while the other three lines were resistant, as were normal cells to AOA. However, three resistant lines (but not normal cells), became sensitized to undergoing apoptosis when TRAIL was combined with AOA. TRAIL by itself had minimal effects on all cell lines and normal cells, and did not augment AOA-induced killing in the two sensitive melanoma lines. AOA plus TRAIL induced a caspase-dependent apoptotic response. AOA did not influence TRAIL DR4 or DR5 cell surface death receptor levels, but AOA enhanced pro-apoptotic protein levels of Noxa, while reducing pro-survival protein Mcl-1. To verify AOA was targeting glutamine pathway, depletion of glutamine produced similar results, because absence of glutamine sensitized three melanoma lines, but not fibroblasts to killing by TRAIL. Glutamine depletion also led to Noxa induction. These results indicate some lines are addicted to glutamine, and treatment with AOA or glutamine depletion sensitizes melanoma to TRAIL-mediated killing, while sparing normal cells. Future studies are indicated to translate these discoveries to metastatic melanoma as there is currently no treatment available to prolong survival.

1. Introduction

Glutamine metabolism, like glucose metabolism is abnormal in cancer cells; and glutamine usage in cancer cells is very inefficient [1]. Even though glutamine is the most common amino acid, no previous reports focus on glutamine metabolism in melanoma cells from a therapeutic perspective as regards using antagonists combined with death receptor ligands. Metastatic melanoma is a highly aggressive malignancy, and is nearly always fatal, with a median survival of only 6–9 months once distant sites become seeded from skin [2]. While dacarbazine (DTIC) is the only FDA approved chemotherapeutic agent for metastatic melanoma (a tumor response of 5–20%), many other therapeutic approaches have been tried, including multi-agent chemotherapy, radiation therapy, and immunotherapy. One important obstacle in identification of therapeutic approaches in the past has been the relatively poorly understood biochemical pathways that govern proliferation and survival pathways of melanoma cells. Given rapid progress in understanding of the biology of melanoma cells, new opportunities have arisen for investigative skin biologists and clinicians to attack this deadly neoplasm [3].

Because of the notorious difficulty in killing melanoma by targeting a single pathway, it is reasonable to consider using a combination of agents that target different pathways to achieve enhanced beneficial results. Recently, a growing body of literature has shifted from conventional emphasis on cell proliferation and resistance to apoptosis of tumor cells to focus on cancer cell metabolomics [4,5]. In this report, we target glutamine metabolism and use a two agent strategy; first focusing on aminooxyacetate (AOA) interfering with glutamine metabolism, and combining AOA with tumor necrosis factor-related apoptosis inducing-ligand (TRAIL). To further emphasize the glutamine addiction pathway, cells were also studied using medium depleted of glutamine.

As there are two major apoptotic pathways (i.e. so-called extrinsic or direct/death receptor-mediated, and the intrinsic/indirect or mitochondrial-based) responsible for mediating death of tumor cells [6], we sought to determine if enhanced killing of melanoma cells addicted to glutamine could be accomplished by activating the extrinsic death pathway. Activating the extrinsic death pathway using TRAIL in combination with AOA, overcame the apoptotic resistance of several of the melanoma cell lines; while leaving two different normal cell types (e.g. melanocytes and fibroblasts) unscathed. Enhanced killing by AOA plus TRAIL was mediated by caspases because a pan-caspase inhibitor

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(e.g. ZVAD) reversed the cell death, and also involved damaging the outer mitochondrial membrane with increased permeabilization and induction of Noxa.

Taken together, the current findings indicate that overcoming the notorious resistance of melanoma to conventional therapy is achievable, by combining AOA together with TRAIL, or by using glutamine depletion plus TRAIL [7]. Further studies are warranted as regards altered melanoma metabolomics and new therapeutic agents targeting glutamine metabolism are emerging and are likely to be exploited for consideration in future clinical trials [8].

2. Materials and methods

2.1. Cell cultures and reagents

Five human melanoma cell lines OCM1A, Mum2C, A375, UACC3093, and c8161 cells were grown in either DMEM medium (OCM1A, A375 cells), or RPMI-1640 medium (all other cell lines). Both conventional and glutamine-free RPMI-1640 medium and DMEM medium were purchased from Lonza (Walkersville, MD) and supplemented with 10% FCS (Gemini Bio-Products, Woodland, CA). Normal adult human skin-derived melanocytes were purchased from Lonza, and grown in the medium provided by the company as previously described [9]. AOA and propidium iodide were purchased from Sigma (St. Louis, MO). Both recombinant human TRAIL and Annexin-V–FITC were obtained from Biovision Research Products (Mountain View, CA). Mouse monoclonal abs against TRAIL receptors (DR4, DR5) and fibroblasts were used as previously described [10]. All cells were maintained in a humidified incubator under standard conditions (37 °C, 5% CO_2). To detect and quantify cell death receptor expression, melanoma lines were treated with AOA (1 mM) for 24 h. After harvesting, cells were incubated with specific abs detecting human DR4 or DR5 at 5 μg/ml for 1 h. Cells were washed with PBS and incubated with Alexa-488 labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for 0.5 h followed by FACS analysis.

2.2. Cell treatment and assessment of cell death

Cells were plated into 24-well plates overnight, and on the second day cells were pre-treated with AOA at different concentrations (0.1, 0.5, 1 mM) for 0.5 h followed by adding recombinant human TRAIL (100 ng/ml) for 24 h. Deprivation of glutamine was accomplished by washing cells with PBS, and replacing conventional medium with glutamine-free medium (Lonza) plus 10% dialyzed FCS. TRAIL was added at 1 h after cells were maintained in glutamine-free medium. Cell death was assessed by flow cytometry after staining with Annexin-V–FTIC and 1 μg/ml propidium iodide (Sigma). Both Annexin-V positive, and Annexin-V positive plus PI positive, or only PI positive cells, were considered as dead cells.

2.3. Mitochondrial membrane potential assay

Cells were harvested (either untreated or treated) and incubated with fresh medium containing 5 μg/ml of Rhodamine 123, a fluorescent dye that selectively accumulates in mitochondria of living cells with normal $\Delta\psi_m$ for 0.5 h. Cells were washed with PBS to remove excess probe, and analyzed by FACS. Reduction of Rhodamine 123 fluorescence intensity indicates loss of mitochondrial membrane potential.

2.4. Immunoblot assay

Cells were harvested by scraping and lysed with M-Per Mammalian Protein Extraction Reagent (Thermo Scientific, Rockville, IL) supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH) and phosphatase inhibitor cocktail set II (Calbiochem, Los Angeles, CA), followed by shaking and centrifugation at 4 °C. Supernatants were collected as whole cell extracts and protein concentrations were measured using Bradford reagents (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein were resolved by SDS–PAGE and transferred to PVDF membrane followed by 1 h blocking buffer supplied by Li-COR Biosciences (Lincoln, NE). Blots were probed with primary abs overnight at 4 °C, washed and incubated with fluorescence-labeled secondary abs for 1 h at room temperature in dark. Protein levels on the membrane were visualized with Li-COR Infrared Imaging System. Abs used were obtained as follows: caspase 3, GAPDH, Mcl-1, Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal ab against Noxa and caspase 8 were from purchased from Calbiochem (San Diego, CA) and Fischer Scientific (Pittsburgh, PA), respectively. Ab against Bid was purchased from Cell Signaling (Beverly, MA).

2.5. RT-PCR

Total RNAs were isolated from cells with TRIzol (Invitrogen). One microgram of total RNA was converted to cDNA using reverse transcription reagents (Applied Biosystems, Foster City, CA). PCR was performed with platinum Taq DNA polymerase (Invitrogen). The following specific primer pairs were used: Noxa: 5-AGA TGC CTG GGA AGA AG-3 (forward), 5-AGT CCC CTC ATG CAA GT-3 (reverse); Mcl-1: 5-CGG TAA TCG GAC TCA A CCTC-3 (forward), 5-CCT CCT TCT CCG TAG CCA A-3 (reverse); 18s RNA: 5-GGC GCC CCC TCG ATG CTC TTA G-3, (forward), 5-GCT CGG GCC TGC TTT GAA CAC TCT-3 (reverse). PCR products were separated by electrophoresis using 1.5% agarose gels and visualized with ethidium bromide.

2.6. Statistical analysis

Statistical analysis was performed using unpaired, two sided Student's *t*-test, and results considered significant when p values <0.05.

3. Results

3.1. Differential AOA-mediated cytotoxicity of melanoma lines, melanocytes and fibroblasts in the absence and presence of TRAIL

The sensitivity of melanoma lines to AOA alone (0.1, 0.5, and 1 mM, 24 h) or TRAIL alone (100 ng/ml), or combination of AOA plus TRAIL, revealed three melanoma lines (A375, c8161, and UACC3093 cells) relatively resistant to killing by AOA alone (Fig. 1A), while two melanoma lines (OCM1A and Mum2C cells) were killed by AOA alone (Fig. 1B). The sensitivity of melanoma lines to TRAIL alone revealed increased killing ranging from approximately 10% to 40% among the AOA resistant cell lines (Fig. 1A); but complete resistance in the AOA sensitive lines (Fig. 1B). Interestingly, when AOA and TRAIL were combined, a concentration-dependent enhanced killing by TRAIL occurred when AOA was present at 0.5 and 1 mM concentrations (Fig. 1A, double asterisks). However, in two cell lines (OCM1A and Mum2C cells), co-presence of AOA and TRAIL did not enhance overall killing (Fig. 1B). To examine potential cytotoxicity for AOA and TRAIL on normal cells, similar experiments were performed using melanocytes and fibroblasts (Fig. 1C). Neither AOA alone (1 mM) or TRAIL alone, or combination of AOA plus TRAIL triggered significant killing of normal cells. Thus, combining AOA plus TRAIL showed promise as we could achieve significant killing (>60% of total population) in three different melanoma cell lines, without harming two different normal cells.