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Methionine γ -Lyase 2-AminoButyrate Deaminase (MEGL-2ABD) as a Gene Therapeutic Agent for Cancers

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Abstract

Methionine is a key nutrient that is required for many metabolic processes/pathways. In bacteria methionine is cleaved into methanethiol and 2-aminobutyrate which is deaminated into α-ketobutyrate and ammonia by methionine γ-lyase 2-aminobutyrate deaminase (MEGL-2ABD); an enzyme that is absent in mammals. We have molecularly cloned *Porphyromonas gingivalis,* DNA (FJ875028) of MEGL-2ABD gene into pEGFP-C3 mammalian expression vector and have transfected the construct into various cancer cell lines such as Hela, HEK-AD293T, BHK-21, methionine dependent prostate PC-3, and independent DU-145 cancer cell lines. Confocal microscopy evinced two interesting observations that occur under methionine deprivation due to MEGL-2ABD. 1. Severe cell aggregation and 2.Cell death perhaps due to apoptotic signaling. Both of these processes are reversed by propargylglycine, an inhibitor of MEGL-2ABD. The severity of cell aggregation or cell death varied depending on the cell types. When MEGL-2ABD gene therapeutic agent was compared to existing anticancer drugs such as methotrexate, MEGL-2ABD had about equal cell death measured by MTT assay. Combination of MEGL-2ABD, along with methotrexate, AraC and vesicular stomatitis virus (VSV) had much higher cell death in prostate cancer PC3 and DU145 cell lines. Exogenously, MEGL-2ABD treated culture media had same effects to that of transfected cells. Thus, cytoplasmic localization of MEGL-2ABD has great potential as a gene therapeutic agent to control cancer cell division.

Keywords: L-Methionine γ-Lyase/L-2-Aminobutyrate Deaminase; Cancer cell metabolism; Methionine metabolism; Sulfur amino acids; Cancer cell lines

Abbreviations: MEGL/2ABD: L-Methionine γ-Lyase/L-2-Aminobutyrate Deaminase; GFP: Green Fluorescent Protein

Introduction

Methionine is one of the essential amino acids that is required during growth and developments. From diet methionine can be absorbed through the intestinal epithelium and can be used in various anabolic pathways. Particularly, methionine is converted into s-adenosylmethionine (SAM) which is the universal methyl group donor. There are several molecules that can be methylated such as DNA, histones, mRNA, ethanolamine, etc. Methylation of DNA CpG islands and histone lysine residues to form trimethyllysine are key factors that contribute to the epigenetic mode of inheritance [1-3]. The DNA and histone specific methylation patterns are quite different between normal and cancer cells. The expressions of specific DNA methyltransferases (DNMT's) and histone methyl transferases are temporally and chronologically programmed during growth and developments. Deregulation of these methyltransferases can lead to cancer cell progression (metastasis) or cancer cell death [4,5]. Once methyl group is transferred as part of the methyl transfer reaction the remaining moiety of s-adenosylhomocysteine is metabolized into homocysteine. Homocysteine through condensation with serine forms cystathionine catalyzed by cystathionine β-synthase [6-8]. Cystathionine through a trans sulfurylase lytic reaction catalyzed by cystathionase forms the nonessential amino acid cysteine and the deaminated product α-ketobutyrate. Homocysteine is also converted back to methionine through a resynthetic enzyme methionine synthase (5-methyltetrahydropteroyl-L-glutamate: L-homocysteine S-methyltransferase) using coenzymes methylcobalamin (vitamin B12) and N5-N10 methylene tetrahydrofolate (vitamin B9). The requirements of vitamins B9 and B12 as well as the levels of methionine synthase are quite different between normal and cancer cell types for cell division [9-11].

In bacteria, methionine is used in the anabolic pathway after modification into formyl-methionine-tRNA (fmet-tRNA). F-met is the first amino acid that gets incorporated into proteins during synthesis [12]. However, the unmodified free methionine that is probably in excess is channeled into catabolic pathways for energy metabolism. Thus, in bacteria as part of the catabolism, the free methionine (met) is cleaved into methanethiol and 2-aminobutyrate by the lyase activity of the methionine γ-lyase 2-amionobutyrate deaminase (MEGL-2ABD) [13-16]. In the second step, 2-aminobutyrate is deaminated into α-ketobutyrate and ammonia by deaminase activity of the bifunctional enzyme MEGL-2ABD. The product α-ketobutyrate itself can further be completely metabolized to yield energy. The direct use of methionine as an energy source and the corresponding enzyme MEGL-2ABD is absent in mammals.

We have molecularly cloned MEGL-2ABD from *Porphyromonas gingivalis* into pEGFP-C3 mammalian expression vector and have transfected the construct into various cancer cell lines. Upon MEGL-2ABD *in vivo* activity and due to methionine deprivation, we hypothesized that the associated pathways such as SAM, cysteine, glutathione, protein synthesis and the overall methylations of DNA, histones and mRNA (7methyl-G) capping will be affected. In this article we report the effects of MEGL-2ABD on various cancer cells that either

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causes severe cell aggregation and/or cell death due to methionine deprivation. Thus MEGL-2ABD could be a potential cancer target.

Materials and Methods

Materials

Oligonucleotides were custom made at the DNA core facilities of John's Hopkins University. Taq DNA polymerase and reagents for cloning were purchased from Invitrogen Inc. Cell culture reagents were purchased from Invitrogen Inc. All other compounds, solvents and reagents were purchased from Sigma-Aldrich. Tissue culture medium and supplies are purchased from Life Technologies Inc.

Cloning and expression of MEGL in mammalian cells and confocal microscopy

The recombinant vector pMEGL-2ABD-ET containing MEGL-2ABD insert and pEGFP-C3 mammalian expression vector (Promega, Inc.) were digested with EcoR I and Bgl II restriction enzymes. The digested products were separated on 1% agarose to verify digestion and the MEGL-2ABD insert was ligated into pEGFP-C3 vector. The ligated vector was then transformed into DH5α bacteria and the recombinant colonies were selected by kanamycin resistance. The plasmid from the recombinant colonies, were further tested for the insert by PCR using MEGL-2ABD specific primers. The recombinant bacteria, was then grown in bulk, plasmid isolated by maxi/mini preps using columns (Qiagen, Inc.). Purified plasmid was then used for mammalian transfection. HeLa, HEK-AD293T and BHK-21 cells (ATCC, Rockville, MD) were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen Inc), in an atmosphere of 5% CO_2, 95% air at 37°C. For confocal microscopy, cells were plated on cover slips in 35 mm^2 dishes. When cells reached 50-75% confluency they were placed in serum and antibiotic-free medium and co-transfected with plasmid DNA containing GFP and with or without MEGL-2ABD insert. Transfections were achieved with LipofectAMINE® 2000 (Invitrogen, Inc.) according to manufacturer's instructions. Transfections of HEK-AD293T cells were performed using standard calcium phosphate methods. After incubation of cells for 4-18 h, cells were washed with phosphate-buffered saline, fixed with 4% formaldehyde for 8 min, permeabilized using 0.2% Triton X-100 (Sigma-Aldrich) and mounted on glass slides using the ProLong Antifade Kit (Molecular Probes) for staining with DAPI using standard protocols. Samples were examined on a Zeiss LSM510 confocal microscope.

Cell cuture for cell viability assay

Prostate cancer cells DU-145 (methionine independent) and PC-3 (methionine dependent) were grown in RPMI medium (Invitrogen Inc.) until confluency. Once ready for transfection cells were trypsinized, washed and re-suspended in optiMEM media. 200 µL of cell suspension (1×10^4) was added onto 96 well plates for MEGL-2ABD transfections. For depleting the methionine contents of the opti-MEM media, homogeneous MEGL-2ABD protein was added to a final concentration of ~0.625 µM and incubated at 55°C for 1 h. After the MEGL-2ABD treatment the respective media were filtered and the sterile media was used for transfections. Transfections were typically done for 18 h at an approximate cell confluency of 75-80%. Cells were processed for MTT cell viability assay using standard procedures.

Cell viability assay

The effects of MEGL-2ABD on cell viability on prostate cancer cells (DU-145 and PC-3) were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). For MTT assay, cells were suspended at a concentration of ~ 4×10^4 cells (180 µl/well). 20 µl of MTT solution (5 mg/ml) was added for each well and incubated at 37°C and 5% CO_2 for about 2-5 h for purple color development. Formazan precipitates were dissolved in DMSO for 30 min at 37°C, and the absorbance was measured at 570 nM.

MEGL transfection and VSV infection

For VSV experiments, prostate cancer cell lines (DU-145 or PC-3) were grown on 96-well plates to ~60% confluence. Using lipofectamine, cells were transfected with plasmid (5 µg /15 ml media) of the gene that encodes for MEGL-2ABD or empty plasmid without MEGL-2ABD gene insert. After 24 h of transfections cells were washed with 2 ml of PBS. Washed cells were then incubated for additional 24 h with a) media treated exogenously with MEGL-2ABD protein or b) non MEGL-2ABD protein treated media c) and/or propargylglycine at different concentrations in the media. Following incubations, cells were infected with VSV-GFP (gift from Dr. Glen Barber, University of Miami Sylvester Cancer Center) at an MOI of 1.0 for 36 h. Cell viability was assayed after infection using MTT method as described above.

Results

Mammalian Cell Expression of MEGL-2ABD

The MEGL-2ABD cloned into mammalian pEGFP-C3 vector was transfected into HeLa, HEK-AD293T and BHK-21 cells. Significant transfection was seen within 4 h as evidenced by GFP expression (data not shown). Cell counts were similar in both control (GFP alone) and experimental (GFP+MEGL-2ABD), 4 h after transfection as evidenced by DAPI staining and MTT assays (data not shown). However HeLa cells after 18 hours of transfection, GFP+MEGL-2ABD construct showed significant cell death as seen by confocal images of GFP and DAPI staining. Proparglycine is a potent inhibitor of MEGL-2ABD as we have shown with the purified enzyme kinetics (data not shown). Treatment of cells with 10 µM propargyl glycine 2 h after transfection in the culture medium restored nearly all cells from death (Figure 1). HEK-AD293T (human adherent kidney) and BHK-21 (baby hamster kidney) cells transfected with MEGL-2ABD constructs showed very prominent cell aggregation. Treatment of cells with 10 µM propargyl glycine 2 h after transfection in the culture medium inhibited the cell aggregation that is prominently evident as shown by the confocal images (Figure 1).

Cell viability

Cell viability measured by MTT assay showed 32 (+/-2.2) % of Hela cell death. Interestingly, cell viability measured by MTT assay showed no cell death or very little cell death if any of HEK-AD293T and BHK-21 cell lines (Figure 2). Prostate cancer cell lines (Figure 3) methionine independent (DU-145) and methionine dependent (PC-3) cells were transfected with MEGL-2ABD or the culture media that was treated with exogenous MEGL-2ABD. Methionine independent DU-145 cells did not show much of cell death (<10%) with transfected (*in vivo*) or culture media treated (exogenous) MEGL-2ABD. Propargylglycine at 30 µM restored moderate cell death observed with DU-145. In PC-3 there were >20% cell death with MEGL-2ABD transfection (*in vivo*) or media treatment with exogenous MEGL-2ABD. Propargylglycine at 0.30 µM in culture medium restored some cell survival whereas in the

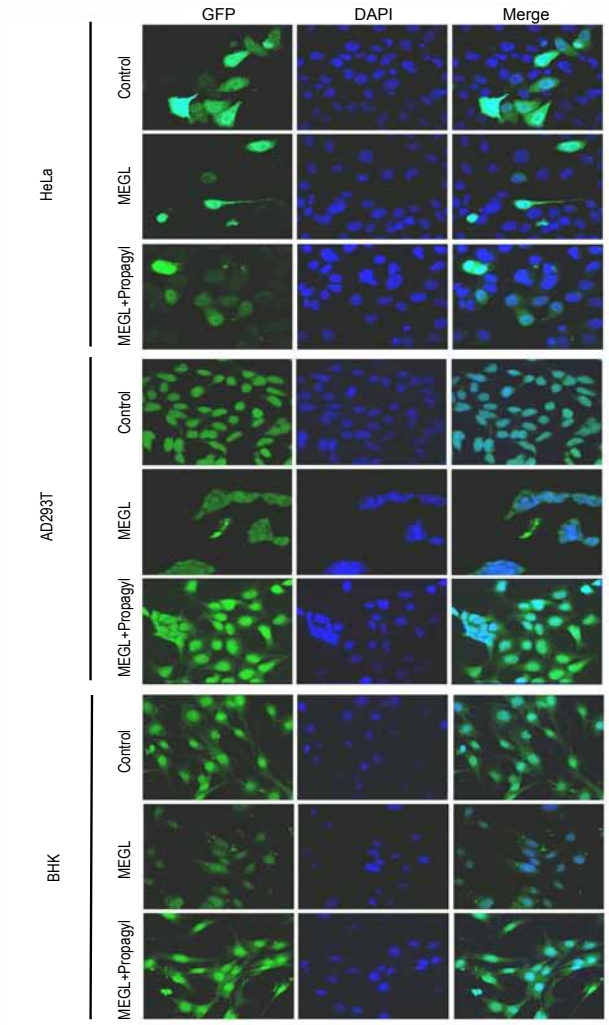

Figure 1: HeLa, HEK-AD293T, BHK-21 cell transfection with pEGFP-C3 vector alone or pEGFP-C3–MEGL-2ABD constructs. MEGL constructs+propargylglycine and its effects on cells were also studied.

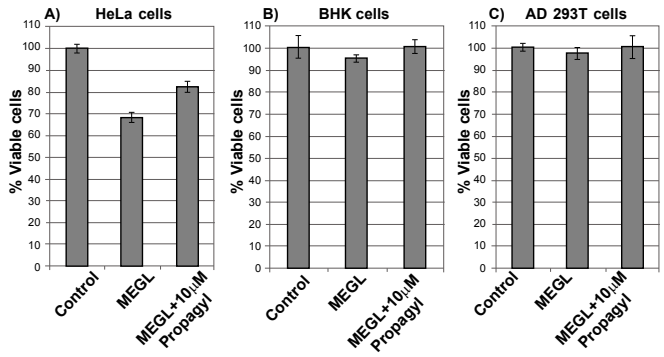

Figure 2: Effects of MEGL-2ABD transfections on Hela, BHK and AD293T cells. Cell viability was assessed by MTT assay. The data represents replicates of 16 wells per cell lines and each experiment was repeated twice.

presence of 30 μM propargylglycine it restored nearly all of the cells from death caused by MEGL-2ABD transfections/treatments. PC-3 cells transfected with MEGL-2ABD, treated with exogenous N-formyl-methionine (f-met) at 1 mM caused about 20% cell death. This cell death did not happen in cells that were not transfected with MEGL-2ABD that contained only f-met (1 mM) in the culture media (Figure 3). When we compared effects of existing chemotherapeutic agents such as methotrexate (MTX) and AraC with MEGL-2ABD on DU-145 cells (Figure 4) MEGL-2ABD had ~10% cell death. Whereas MTX at 1 μM level had ~15% cell death compared to AraC (at 1 μM level) that had less than ~10% cell death. When MEGL-2ABD was combined with MTX, it showed >22% cell death, whereas AraC+MEGL-2ABD combination did not show any improvement. Vesicular stomatitis virus (VSV) by itself on DU-145 cells and PC-3 had no effects. However in combination with MEGL-2ABD, PC-3 cells exhibited nearly 50% cell death and DU-145 cells did not have any effect (Figure 5).

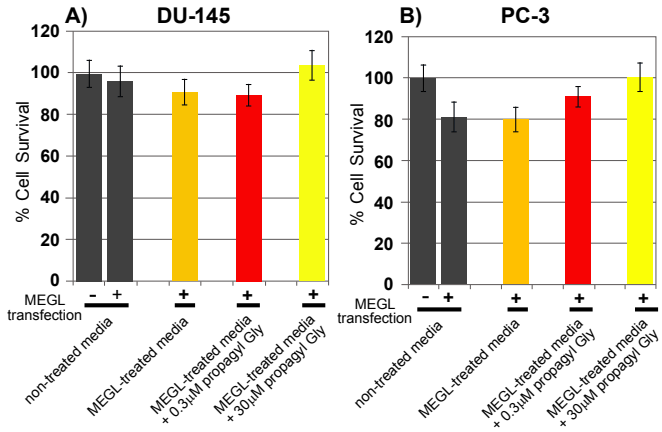

Figure 3: Effects of MEGL-2ABD on Methionine independent (DU-145) and methionine dependent (PC-3) prostate cancer cell lines. Experiments were performed with replicates of 16 wells per condition and each experiment was repeated 4 times.

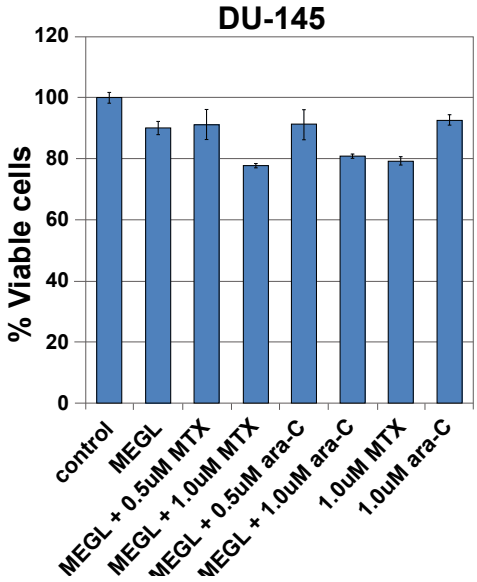

Figure 4: Effects of methotrexate (MTX), AraC and MEGL-2ABD on methionine independent prostate cancer cell line (DU-145). Experiments were performed in replicates of 8 wells per condition and each experiment was repeated twice.

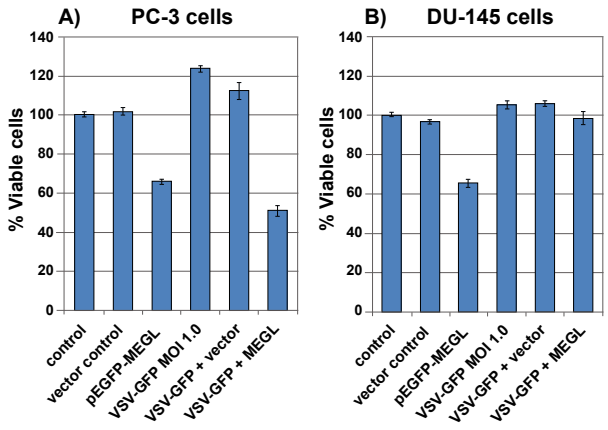

Figure 5: Effects of VSV and MEGL-2ABD on A. PC-3 (methionine dependent) and B. DU-145 (methionine independent) prostate cancer cells. Experiments were performed in replicates of 8 wells per condition and each experiment was repeated thrice.

Discussion

Methionine is an essential amino acid that is normally obtained in humans through diet. Methionine is required for SAM, cysteine, glutathione and eukaryotic protein synthesis. Overall, methylations of DNA, histones and mRNA (7methyl-G) capping in the nucleus are dependent on s-adenosyl methionine (SAM)/methionine. The intracellular concentration of methionine has been reported to be ~1 mM. Deprivation of methionine in cells would hamper many of the metabolic processes/pathways that are crucial to cell division/cell progression. We have demonstrated in test tubes that methionine treated with cloned, expressed and purified MEGL-2ABD at a final concentration of ~0.625 μM can degrade methionine at (~1 mM) completely into methanethiol and α-ketobutyrate within 1 h between 37-55°C (data not shown). We have transfected the bacterial (*Porphyromonas gingivalis*) MEGL-2ABD gene into various cancer cell lines. This is the first report to show the *in vivo* effects of GFP fused MEGL-2ABD on various cancer cell types. MEGL-2ABD caused cell death that ranged anywhere between 10%-40% depending on the cancer cell types, the metabolic states (methionine dependent versus methionine independent) and transfection efficiencies between experiments. Exogenously added MEGL-2ABD on cell culture medium had cell death effects similar to that of *in vivo* transfected MEGL-2ABD proving methionine depletions and deprivations can ultimately affect cancer cell metabolism.

As evinced by confocal microscopy certain cancer cells such as BHK and AD293T exhibited severe cell aggregation due to *in vivo* pEGFP-C3-MEGL-2ABD compared to control cells that was transfected only with pEGFP-C3. Perhaps, the cells are forced into quiescence pathway due to methionine deprivation. The inhibitor of MEGL-2ABD completely reversed the cell aggregation to an extent of cells looking even brighter on confocal images. Whereas Hela cells did not show cell aggregation and it underwent cell death. Thus, BHK and AD293T cells must be sensing the nutrient availability much better compared to Hela cells. Thus in contrast to Hela cells, BHK and AD293T cells must be avoiding apoptotic signaling mediated cell death hoping to revive back when the nutritional status becomes normal.

Aside from the dietary source of methionine, preexisting intracellular homocysteine can be converted back into methionine through a resynthesis pathway catalyzed by methionine synthase. Methionine synthase [5-methyltetrahydropteroyl-L-glutamate: L-homocysteine S-methyltransferase] using coenzymes methylcobalamin (vitamin B12) and N5-N10 methylene tetrahydrofolate (vitamin B9) converts homocysteine into methionine [11,17]. The activity of this enzyme is quite different between methionine dependent and independent cells. Methionine independent prostate cancer cell DU-145 apparently has more of resynthesis ability compared to methionine dependent prostate cancer cell line (PC-3) [18]. In all our experiments MEGL-2ABD had much pronounced cell death effects on PC-3 relative to DU-145 a reason that fits very well with the a priory methionine dependency/independency factor. Propargylglycine reversed the MEGL-2ABD effects on the prostate cancer cell lines asserting the specificity of the MEGL-2ABD. Metabolically cancer cells are very diverse and it can adjust to the adversity and can undertake a path that is quite unique i.e., rapid cell division and consequent cell death, or slower cell division or no cell division such as quiescence. F-met is the preferred form of methionine that is required by the bacteria for protein synthesis. F-met perhaps gives stability to the proteins or confers specific signature recognition to the prokaryotic ribosome initiation factor during mRNA translation [12]. We are the first one to prove *in vitro* that f-met is not degraded by MEGL-2ABD and formyl group actually confers protection (data not shown). When f-met was substituted in the culture media the MEGL-2ABD transfected cancer cells (DU-145 and PC-3) did not show any improvements on cell survival compared to control cells that had only f-met in the media or had no MEGL-2ABD transfection. This is quite interesting that f-met cannot substitute for free unmodified methionine in eukaryotes. Cancer cells even under methionine deprived extreme conditions could not use f-met for its metabolism. Thus, there is a clear distinction between prokaryotes and eukaryotes in terms of methionine incorporation into protein during initial synthesis. Albeit, f-met not being a substrate for protein synthesis in eukaryotes many proteins are acetylated at the N-terminal methionyl residue as part of the post translational modification to protect eukaryotic proteins from N-terminal degradation. VSV is a virus that specifically infects cancer cells as opposed to normal cells [19-22]. When added to infect PC-3, VSV caused significant cell death. However, in DU-145, VSV did not have any effects on cell death. When VSV was combined with MEGL-2ABD transfections both cell types exhibited cell death, more pronounced on PC-3. Thus VSV-MEGL-2ABD might even be a better tool to target cancer cell death. When multi-pronged cancer therapeutics is used as a target, perhaps, we may better be able to control cancer cell survival and its progression of various cancer cell types. We are currently in the process of localizing MEGL-2ABD in the nucleus with the hope that methionine/SAM dependent metabolism can be curtailed at the very early stages of cancer cell gene expression by preventing mRNA (7methylG) capping.

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