CD73 molecule inhibitor upregulates miR16 expression in experimental glioblastoma and inhibits angiogenesis by targeting VEGF

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Abstract

The function of CD73 (cluster of differentiation 73), an enzyme that is involved in adenosine (ADO) generation, in glioblastoma development has been demonstrated. However, the details of its molecular mechanisms have not been fully realized. Due to the significance of angiogenesis in cancer progression, invasion, and metastasis, in this study, it was intended to evaluate the impact of CD73 inhibition by adenosine 5'-(α , β -methylene) diphosphate (APCP) on the angiogenesis process of experimental orthotopic glioblastoma at mRNAs, microRNAs, and proteins levels. According to the real time-poly chine reaction (RT-PCR) findings, CD73 inhibition decreased glioblastoma angiogenesis by reducing vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF-1 α) expression. Furthermore, immunohistochemical staining showed that this treatment protocol attenuated the expression of VEGF and CD31. Moreover, APCP treatment in the glioblastoma model rats significantly increased miR-16 expression, but no significant change in expression in miR-29A was observed. The results identified that the treatment did not induce systemic damage or significant body weight loss. Our findings indicate that CD73 inhibition can diminish tumor new vessel generation by inhibiting the molecules involved in this process, thereby CD73 may be a practical target and provide novel opportunities for improving the treatment of malignant brain tumors.

Introduction

Glioblastoma Multiforme (GBM) is a type of brain cancer that is referred to as the prevalent malignant central nervous system (CNS) neoplasm in adults. Basically, the tumor is very aggressive and spreads rapidly. Furthermore, glioblastoma is extremely resistant to the majority of chemotherapeutic drugs and radiation therapy regimens. Therefore, the identification of novel molecular targets is highly essential to provide treatment choices for these patients [1, 2].

CD73 known as Ecto-5'-nucleotidase (NT5E), an enzyme that is connected to the outside surface of the plasma membrane, catalyzes the conversion of 5'-AMP into extracellular adenosine. CD73 up-regulation was confirmed in varied forms of cancer cell lines and tumor biopsy's patients [3–5]. According to several studies, inhibition of CD73 reduces the proliferation of glioblastoma cell lines and diminishes the promotion of glioblastoma in animal models [6–9]. In addition, inhibition of CD73 enhances the effect of temozolomide chemotherapy in preclinical models of glioblastoma [10]. Furthermore, the elimination of CD73 in tumor cells decreases tumor proliferation, inhibits tumor epithelial-mesenchymal transition (EMT), and increases the antitumor response and the efficacy of cancer immunotherapy [11–14].

Angiogenesis is an emerging feature of cancer, and it is critical to tumor progression and invasion [15]. Adenosine accumulates in solid tumors and along with hypoxia initiates various tissue responses, including the stimulation of the angiogenesis process [12]. In addition, low oxygen levels (hypoxia) upregulate the CD73 and adenosine receptor (AR) expression in cancer cells and likewise, AR signaling promotes tumor cells proliferation and triggers the production of factors related to angiogenesis. It is well demonstrated that HIF-1 α can provoke CD73 expression via direct binding to the CD73 promoter

sequence, thus the hypoxic conditions caused by uncontrolled proliferation of tumor cells can induce HIF-mediated up-regulation of CD73 expression in tumor cells [16, 17]. Of note, HIF-1 α is the main adaptor for VEGF presentation in the tumoral microenvironment, which binds to the hypoxia-response element (HRE) in the VEGF promoter regions. Under hypoxic conditions, the level of HIF1 α increases, which positively modulates the expression of VEGF and other angiogenic factors, improves the consistency of VEGF mRNA and eventually results in tumor new vessels formation [18, 19].

MicroRNAs (miRNAs) are considered a group of non-coding small RNAs that can influence gene expression through disrupting the mRNA expression of the targets [20]. Some special miRNAs are related to hypoxia and hypoxia-associate maturation of endothelial angiogenesis properties. Moreover, they can modulate different aspects of angiogenesis, such as maturation, duplication, and migration of endothelial cells [21, 22]. The miR-29 family control the angiogenic reactions in various tissues, and miR-29a is expressed considerably on the endothelium cell surfaces. According to the different study reports, miR-15a and miR-16 could regulate the angiogenesis process of multiple myeloma owing to controlling the VEGF expression. The upregulation of miR-16 suppresses VEGF molecule generation in decidua-derived mesenchymal stem cells (dMSCs) through targeting its 3' UTR [23]. The miR-16 also indirectly downregulates CD73 expression [16].

The purpose of the current project is to assess the effect of CD73 molecule inhibitor (adenosine 5'-(α , β -methylene) diphosphate (APCP)) on angiogenesis process along with miR-29a/miR-16 expression evaluation in experimental orthotopic glioblastoma.

Materials And Methods

Reagents

Adenosine 5'-(α , β -methylene) diphosphate (APCP) was bought from Sigma company (United Kingdom). APCP was dissolved in PBS containing 20% DMSO. Rabbit Anti-VEGF antibody (orb191500) and Rabbit Anti-CD31 antibody (orb10314) were prepared from Biorbyt (UK) and HRP Conjugated Goat Anti-Rabbit IgG (BA-1054-2) was purchased from Boster (USA).

Cell culture

The rat C6 glioblastoma cell lines were provided by the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The cells were expanded in Dulbecco's Modified Eagle's Medium Nutrient Mixture/ F12 Ham (DMEM/F-12) (Gibco, Grand Island, USA) containing 10% of fetal bovine serum (FBS) (Gibco, Grand Island, USA), antibiotics (Gibco, Grand Island, USA) at 37 °C in a 95% humidified environment and 5% CO₂.

Animals

Male Wistar rats at 12 weeks of age, weighing 250-300 g, were housed in the animal house of Semnan University of Medical Sciences for one week before the experimentation. The temperature was held at 23 ± 2 °C with a humidity of $55 \pm 5\%$ and at a 12:12 h light-dark rotation.

Stereotactic tumor implantation

Glioblastoma induction was performed as explained earlier with some modifications [6, 24]. In brief, C6 rat glioblastoma cells at 80% confluency were dissociated using trypsin solution, washed with cell culture medium, centrifuged, and then re-suspended in the same medium. Afterward, about 5×10^5 cells in a volume of 10 μ L were injected into the right striatum, through a Hamilton micro-syringe connected with a microinfusion pump (stoeling, USA). The injection rate was 1 μ L/min and the coordinates were 0.5 mm posteriorly to bregma, 3.0 mm lateral at a depth of 6.0 mm. This was done after anesthetizing the animals by the injection of intraperitoneal (i.p) ketamine (80 mg/Kg) and xylazine (10 mg/Kg). On days 7, 9, 12, 14, 16, and 18, APCP (400 μ g/200 μ l) or drug solvent (vehicle) (200 μ l) were administered intraperitoneally.

Totally, there were 3 groups including APCP group (tumor induction and APCP administration), Control (tumor induction and drug solvent administration), and Normal group (without tumor induction and any administration, intact) (Figure 1). One day following the final injection, the rats were weighed, sacrificed and brains were harvested.

Bioinformatics Analysis

The miRNAs involved in the angiogenesis of GBM and genes targeted by the miRNAs were detected via bioinformatics analyses.

Genes contributing to the angiogenesis were extracted from the KEGG (<https://www.genome.jp/kegg>) and the WikiPathways databases (<https://www.wikipathways.org>). The specific target genes of the miR-16-5p and miR-29a were taken from different databases (TargetScan, Protein Atlas, MirDB, MirTarBase, and Unigene databases).

Interactions between the target genes were further taken from the STRING database (<https://string-db.org>).

RNA extraction, Complementary DNA (cDNA) Synthesis, and Real-Time Polymerase Chain Reaction (RT-PCR)

The RNA extraction procedure of glioblastoma tumor tissue was performed by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's suggested method.

5 μ g of total RNA of each sample with Revert Aid™ First Strand cDNA Synthesis Kit (Takara, Japan) was converted to cDNA. Moreover, the reverse transcription of miRNAs was accomplished by BonmiR kit protocol (cat NO. BN-0011.17).

Real-time PCR was run by SYBR Green qPCR Master Mix (cat. no. YT2551, Ykta tajhiz Azma). Primer designing for mRNA was performed with Oligo 7 software and primer 3. The quality of the primer sequence was investigated by BLAST online software. Table 1 shows the sequence of the used primers for RT-PCR amplification obtained here. B2M and U87 were implicated as internal controls for mRNA and miRNAs genes, respectively. The Real-Time PCR program had 3 steps consisted of denaturation at 95° C for 15 s, annealing at 61° C for 30 s, and finally extension at 72 ° C for 30 s. In addition, the thermocycler program for miRNA amplification included a denaturation step at 95° C for 2 min, followed by 35 cycles of the program at 95° C for 15 s, and then at 60° C for 30 s.

Table1 - Primers used in qPCR for mRNAs in Wistar rats

Sequence	Name	Size (bp)
B2m	F: 5'- GCTTGCCATT CAGAAACTCC-3'	74
	R: 5'-GGAAGTTGGGCTTCCCATTCT-3'	
veg f-a	F: 5'- TCGGAGAGCAACGTC ACTA-3'	109
	R: 5'- TCTTTGGTCTGCATT CACATC-3'	
hif-1a	F:5'- GCAGCGATGACACGGAAAC -3'	129
	R: 5'- TTTCAGAGGCAGGTAATGGAGA -3'	

Immunohistochemistry

Paraffin-embedded sections of tumor tissues were warmed at 60° C for 30 minutes and subsequently, rehydrated with xylene and graded alcohols. The slides were heated using TBS 1X buffer for 15 min in a microwave oven in order to antigen retrieval procedure. Using PBS comprising 0.3% hydrogen peroxide, was stopped the endogenous peroxidase activity for 10 minutes. Then, the sections were incubated overnight with rabbit anti-VEGF and anti-CD31 mAbs. Subsequently, washing with PBS, slides were covered with HRP-conjugated secondary antibody for 1 hour to distinguish the antigen-antibody complexes. Afterward, the sections were stained with a di amino-benzidine tetrachloride (DAB) solution. At that point, the slides were hematoxylin contrast stained, dehydrated, and sealed. Finally, the stained slides were analyzed using an optical microscope (LABOMED, USA) and Image J software.

ALT and AST

To determine the systemic toxicity of the drug administration, the level of liver enzymes was measured by a serum biochemistry assay. To do this, about 5 microliters of blood was obtained from the heart of all animals at the time of sacrifice and centrifuged at 3000 rpm for 10 min in order to serum collection. Then, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were analyzed according to the manufacturer's recommendation (Paadco, Golestan Technology Park, Iran).

Statistical analysis

Student's t-test or One-way analysis of variance (ANOVA) was done, and after that Tukey Kramer's post-test (for multiple comparisons), as the case may be, for statistical differences was applied. The GraphPad Prism (version 8) for statistical analysis and graph generation was used. For p-values of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, the differences were considered significant. All statistics were represented as mean $\pm$ standard error of the mean (SEM).

Results

The effect of CD73 molecule inhibitor (APCP) was analyzed in an animal glioblastoma model. After 7 days of tumor implantation, rats were treated with APCP for 12 days (400 $\mu\text{g}/200\mu\text{l}$). Drug solvent-treated animals were considered as the control. On the 19th day of the treatment protocol, rats were scarified and tumoral tissues were evaluated as mentioned in the Methods section (Figure 2). The brain of the intact group (without tumor and treatment) was also removed for comparison with treated groups (Figure 3).

Bioinformatics Analysis Results

The Wiki Pathways, the KEGG databases, and some related articles were used to detect genes involved in the angiogenesis of GBM. Based on our data VEGF signaling promotes angiogenesis by increased cell proliferation (PI3K/AKT/mTOR and ERK1/2), cell migration p38 and released VEGF endocrine. CD73 (Nte 5) triggers angiogenesis by the VEGF signaling pathway; PI3K/AKT/mTOR pathway regulation of HIF-1 α . It induced VEGF, and as a result induces angiogenesis. Then, the miRTarBase, PicTar, and TargetScan databanks were used to characterize the possible target genes of the miR-16-5p and miR-29a. Finally, the interactions between the miRNAs and the selected potential target genes using the STRING database are shown in Figure 4.

APCP reduced VEGF-A and HIF-1 α gene expression

As presented in Figure 5, post-treatment with APCP in the glioblastoma model rats significantly decreased the levels of VEGF-A and HIF-1 α mRNAs at the doses of 400 $\mu\text{g}/\text{kg}$ of APCP ($P < 0.01$ and $P < 0.05$, respectively), compared with the drug solvent-treated glioblastoma model rats (control group).

APCP changed the expression of miR-16 and miR-29a

As displayed in Figure 6, post-treatment with APCP in the glioblastoma model rats significantly increased the expression of miR-16 at the doses of 400 $\mu\text{g}/\text{kg}$ of APCP ($P < 0.05$), versus the drug solvent-treated rats (control group). but, there was not a significant change in miR-29A expression in comparison to the control group.

APCP decreased expression of angiogenic factor proteins

The expressions of VEGF and CD31 were determined in tumor samples obtained from the rat brains (control, APCP, and non-treated animals) by immunohistochemistry assay. Histologic analysis revealed the effective down-regulation of these angiogenic factors inducing elements in the APCP group in contrast to the control. It was also clearly shown that the expression of VEGF and CD31 in both treated groups (control and APCP) were higher than in the untreated group (Figure 7).

Treatment with APCP did not cause systemic toxicity in glioblastoma-bearing animals

For evaluation of the treatment with CD73 inhibitor safety in glioblastoma-implanted rats, liver enzyme measurements were performed. As displayed in Table 2 no alterations were observed in serum ALT/AST activities after treatment between the treated groups and non-treated rats, demonstrating that the treatment did not change the liver function.

Moreover, C6 cells administration induced significant weight loss after the day 9. In contrast, none of the APCP treated rats displayed significant weight loss (Figure 8). It means that the administration of APCP inhibited tumor-induced weight loss.

Table 2. Liver damage markers (AST and ALT) were determined in blood serum with commercial kits. Values are presented as the mean ± SEM from 3 samples and were analyzed using one way ANOVA followed by post-hoc comparisons (Tukey’s test).

Values	Control	APCP	Non-treated
AST (U/L)	83.58±12.45	83.10296±32.75406	73.78±8.94
ALT (U/L)	24.02±13.05	28.31±22.55	20.97±1.25

Discussion

In the tumor microenvironment (TME), different cells, including normal and tumoral cells, release nucleotides (ATP and AMP) when subject to stress like injury, hypoxia, and inflammation. Subsequently, CD39 converts ATP and ADP into AMP and then CD73 hydrolyze AMP to adenosine. The essential function of the CD73 molecule in tumor development via regulating extracellular nucleotide/nucleoside levels was demonstrated in cells of TME [6]. Regarding the high expression of CD73 in gliomas, it plays a key role in cell proliferation and regulates cell adhesion and interaction between cell-matrix and tumor cells [2, 25]. The connection of adenosine to its receptors including A2A and A2B on cancer cells can promote tumor growth and triggers intracellular pathways which result in expression and production of angiogenesis-inducing mediators such as VEGF, CD31, bFGF, and IL-6 whichever promote the formation of new capillaries to feeding the cancer cells. However, it has been proved that CD73 possesses an angiogenic function in cancer progression, nevertheless there is scare evidence about its molecular mechanisms [26–28]. In addition to CD39/CD73, hypoxia and HIF-1α are involved in the provocation of adenosine production. Likewise, low oxygen condition induces the expansion of blood vessels through stimulating the generation of HIF1-α and tumor-released VEGF [29]. Signaling pathway of VEGF activates

p38 kinase, phosphatidylinositol 3-kinases/AKT kinase/ mammalian target of rapamycin kinase (PI3K/AKT/mTOR), extracellular signal-related kinases 1/2 (ERK1/2), and focal adhesion kinase (FAK) pathways in endothelial cells, which in turn lead to some events consist of proliferation, differentiation, migration, and invasion of these cells [30]. Our bioinformatics analysis reported all interactions of these signaling pathways in Fig. 3.

Some studies showed that tumor-associated CD73 stimulates VEGF production through its enzymatic role (production of extracellular adenosine), while suppression of CD73 by antibody, siRNA, or inhibitor leads to down-regulation of angiogenesis in some tumors including breast tumor, cervical cancer, and melanoma [25, 31–33]. The data obtained here supported early researches that showed APCP (CD73 inhibitor) blockage of CD73 in a tumor model of glioblastoma in rat. VEGF mRNA significantly decreased (Fig. 4), and protein (Fig. 7) expression in the treatment group in comparison to the control group as well as PDGF (CD31) protein expression (Fig. 7). The HIF-1 α mRNA expression was not reduce in response to CD73 inhibition that could be due to the severe hypoxic environments in glioblastoma tumors [34]. It seems that our treatment protocol was safe because it did not cause liver toxicity and significant body loss, while glioblastoma caused severe and fatal weight loss in tumor-bearing animals.

There is evidence that miRNAs are involved in the direction of different features of the angiogenesis process. The miR-16 is often down-regulated in different kinds of cancer and has been shown to be a possible tumor suppressor by targeting the BCL-2 oncogene [35]. The miR-16-5p binds to the 3' UTR sequence of VEGF gene, and it is a direct target of miR-16-5p [36]. The miR-16 and miR-15a inhibit multiple myeloma cells by triggering the growth of endothelial cells and blood vessel formation in vitro. Ectopic expression of miR-15a and miR-16 into multiple myeloma cells can dramatically down-regulate VEGF expression [37]. In vivo also, miR-15a and miR-16 markedly inhibited the growth of human myeloma (RPMI-8226) cell line in an animal model and suppressed tumor angiogenesis [37]. The current study revealed that miR-16 is coupled to the VEGF-A 3'-UTR, and this communication can prevent the VEGF upregulation at mRNA and protein levels in the GBM-treated group.

The miR-29a is overexpressed in endothelium in the hypoxic condition of TME, and it has a critical role in controlling the cell growth, and angiogenic phenotype of endothelial cells [38]. Jih-Yang et al. found miR-29a induced VEGF to expression and angiogenic process in synovial fibroblasts cells and overexpression of miR-29a displayed significant decreases in VEGF and HIF-1 α positive synovial fibroblasts [39]. Moreover, miR29b targets VEGFA suppress angiogenesis in endometrial carcinoma through the PI3K/Akt signaling pathway [40]. Nonetheless, our finding did not show any notable association between miR-29a and VEGF expression. Therefore, further experimental studies are required to investigate the targeted miR-29a in the GB rat model.

In accordance with the results of this examination, it seems that CD73 can be selected as a therapeutic target to prevent tumor growth in glioblastoma through angiogenesis inhibitors and induced miRNAs with anti-angiogenesis properties.

Conclusion

It was concluded that CD73 inhibition decreased glioblastoma angiogenesis and miR-16 expression. First, the effect of CD73 inhibition on angiogenic factors including VEGF and CD31 was investigated. It was found that CD73 inhibitor APCP diminished markedly these factors. In line with this, CD73 down-regulation decreased the expression of VEGF-A and HIF-1 α genes in the tumor tissue. Moreover, APCP could significantly up-regulate miR-16 and non-significantly miR-29a in glioblastoma. Finally, analysis of serum factors related to liver function and body loss percentage showed that this treatment is safe for animal models and recommends CD73 as an attractive option for the management of brain tumors.

Declarations

Ethical Approval

All experiments were conducted in accordance with the guidelines for the safekeeping and practice of laboratory animals. All assays were confirmed by the Ethics Committee of the Semnan Medical University Faculty of Medicine, Iran with IR.SEMUMS.REC.1398.087 certificate number.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Samaneh Arab, Samira Asgharzadeh, and Parviz Kokhaie designed the experiments and supervised the project; **Sahar Ghasemi, Ali Ghanbari, Marjan Bahraminasab, and Mina Heidari** performed the experiments; **Samaneh Arab and Samira Asgharzadeh** analyzed data and prepared the figures; **Samaneh Arab and Samira Asgharzadeh** wrote a first draft of the manuscript. All authors discussed results of the experiments, edited and approved the final version of the manuscript.

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Consent to participate

Not applicable.

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Figures

Figure 1

Schematic of the study design

Figure 2

The experimental schedule and treatment plan

Control

APCP

Figure 3

Representative pictures of tumors

Figure 4

Angiogenesis has a central role in cancer progression through different pathways that regulate angiogenesis in GBM. VEGF signaling promotes angiogenesis by increased cell proliferation (PI3K/AKT/mTOR and ERK1/2), cell migration p38, and releases VEGF endocrine (blue nodes). CD73 (Nte 5) Promoting angiogenesis by VEGF signaling pathway (light green edges). PI3K/AKT/mTOR pathway regulation of HIF-1 α and HIF-1 α induced VEGF, as result, improves angiogenesis (dark green edges). MiR-16-5p and Mir-29a suppressed angiogenesis by target VEGF and HIF-1.

GBM, Glioblastoma Multiforme; VEGF, Vascular endothelial growth factor; PI3K, Phosphoinositide 3-kinase; Akt, /protein kinase B; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; HIF-1, hypoxia-inducible factor 1.

Figure 5

The expression of VEGF-A (A) and HIF-1α (B) in the tumor tissue. B2m was regarded as the internal control. Data are represented as the mean $\pm$ SEM from 4 samples and were evaluated using a student's t-test. **P < 0.01 and *P < 0.05 in comparison to the control group.

Figure 6

The expression of levels of miR-16 (A) and miR-29a (B) in the tumor tissue. U87 was regarded as the internal control. Statistics are expressed as the mean $\pm$ SEM from 4 samples and were analyzed using a student's t-test. *P < 0.05 in comparison to the control group.

Figure 7

Immunohistochemical analysis of VEGF/ CD31 expression in tumor samples derived from rats on day 19 post-treatment. Immunohistochemistry images of VEGF (left) and CD31 (right) protein levels expression in the control group, APCP-treated group, and non-treated group. Immunoperoxidase and haematoxylin nuclear counterstaining; original magnifications: 10X (up row) and 40X (down row) (C) expression percentage of VEGF (right) and CD31 (left) in control, APCP and non-treated groups. Bar charts represent the mean $\pm$ SEM from four samples and were analyzed using one way ANOVA followed by post-hoc comparisons (Tukey's test). **P < 0. 01, ***P < 0.001, and **** P < 0.0001 in comparison to control group and ###P < 0.001 and ####P < 0.0001 versus non-treated group.

Figure 8

Effect of CD73 inhibition on body weight in a glioblastoma model. Body weight (A) and their variation percentage (B) between APCP and control groups. Statistics are shown as the mean $\pm$ SEM from 5 samples. *P < 0.05, **P < 0.01, ***P < 0.001, and **** P < 0.0001 versus control group.