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Original Articles

Delivery of MGMT mRNA to glioma cells by reactive astrocyte-derived exosomes confers a temozolomide resistance phenotype

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ABSTRACT

The glioma-astrocyte interaction plays an important role in tumor microenvironment remodeling; however, the underlying mechanism has not been completely clarified. In this study, we show that glioma cells stimulate normal human astrocyte (NHA) into reactive astrocyte (RAS) in a non-contact manner. Additionally, the amount of O6-alkylguanine DNA alkyltransferase (MGMT) mRNA in exosomes (EXOs) released by RAS was significantly increased compared with that in non-reactive NHA. Importantly, MGMT-negative glioma cells can take up RAS-EXOs and acquire a temozolomide (TMZ)-resistant phenotype via the translation of exogenous exosomal MGMT mRNA both in vitro and in vivo. Our findings illuminate a novel phenomenon that may be a potent mechanism underlying glioma recurrence in which glioma-associated NHAs protect MGMT-negative glioma cells from TMZ-induced apoptosis by the functional intercellular transfer of exosomal MGMT mRNA.

1. Introduction

Malignant glioma (WHO grade III–IV) is the most common and most lethal primary tumor of the central nervous system (CNS) in adults [1]. Diffuse infiltration of tumor cells throughout the brain is a core characteristic of high-grade glioma and is responsible for treatment failure even if maximal surgical resection is performed [2,3]. Although temozolomide (TMZ) has been shown to prolong survival, the treatment response is frequently limited by the progression of drug resistance [4,5]. Thus, identifying potential mechanisms underlying TMZ resistance could provide prospective molecular targets for glioma therapy.

In addition to the drastic alterations occurring at the cellular level, the tumor microenvironment (TME) is also relevant for cancer progression [6]. The TME consists of various non-tumor cell types such as endothelial cells, fibroblasts, and inflammatory cells. In the context of glioma, astrocytes and microglia cells are essential components of the TME. Glioma and non-tumor cells frequently exchange information via gap junctions and tunneling nanotubes, also in contact-independent ways such as the secretion of secretory proteins and extracellular vesicles (EVs) [7–10]. These cells interact with each other to form complicated communication networks, thereby profoundly affecting several cancer phenotypes such as neovascularization and chemo/radioresistance [11]. Recent studies have revealed that cells can mutually communicate via exosomes (EXOs), microvesicles that are released by endosomes and can be taken up by cells via endocytosis. The internalization of EXOs by target cells leads to the release of bioactive molecules including proteins, mRNAs, and non-coding RNAs [12–14].

Although recent advances have concentrated on exosomal microRNAs (miRNAs), some studies reported that exosomal mRNA can be translated into functional proteins in vitro and in vivo [13–18]. Zomer et al. found that exosomal mRNA can be functionally translated into protein in vivo, as evidenced by transfer of CRE mRNA within EVs to a cell carrying a fluorescent reporter gene that is expressed only after CRE-mediated DNA excision of a STOP signal and can be detected by fluorescence changes [15]. Pastuzyn et al. showed that Arc EVs and capsids can mediate intercellular transfer of Arc mRNA in neurons [18]. A growing body of research has suggested that EXOs released by glioma cells modulate surrounding cells to create an optimal environment for cancer development [19–21]; however, the functional roles of astrocyte-derived EXOs in modulating the malignant phenotypes of recipient glioma cells have not been elucidated [14,22].

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Recent reports have shown that mRNAs are functionally secreted in microvesicles.

Shao et al. showed the presence of MGMT mRNA encoding O6-alkylguanine DNA alkyltransferase in patient-derived EXOs and demonstrated that the level of the exosomal MGMT mRNA could predict the response of glioblastoma patients to TMZ [23]. Furthermore, Garnier et al. reported that the MGMT mRNA profile reflective of TMZ resistance was recapitulated in the transcriptome of EVs released by glioma stem cells [17]. Although these studies are beginning to shed light on the importance of exosomal MGMT mRNA in predicting the outcome after TMZ application, the pathological effect of EXOs meditated MGMT mRNA exchange in glioma progression remains unclear. Therefore, we hypothesized that MGMT mRNA embedded in astrocyte derived EXOs might be a potential transmitter to acquire TMZ resistance in recipient cells.

In this study, we showed that NHA (normal human astrocyte) is stimulated by glioma cells, thereby presenting a reactive condition that leads to an increased expression level of MGMT mRNA in both cell and EXOs. Delivery of reactive astrocyte (RAS) MGMT mRNA via EXOs to MGMT-negative glioma cells might transfer TMZ resistance through effective translation of MGMT mRNA.

2. Materials and methods

The Supplementary material section is referred for details on western blotting, RNA extraction, quantitative reverse transcription–PCR, methylation-specific PCR, plasmid construction, transfection, stable cell establishment, flow cytometric analysis, EdU proliferation assay (EdU), CCK-8 assay and immunofluorescence (IF) [24,25].

2.1. Reagents, patient tissue specimens and cell lines

Lipopolysaccharide (LPS) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Temozolomide (TMZ) and GW4869 were purchased from Selleck Chemicals (Houston, TX, USA). This study was approved by the institutional review board and ethics committee of Nanjing Medical University and written informed consent was obtained from all participants. Information of patient samples is detailed in the Supplementary Material. The human GBM cell line U87 was purchased from the ATCC and was validated by short tandem repeat DNA fingerprinting. Normal human astrocytes (NHAs) (ScienCell) were grown in astrocyte basal medium supplemented with 2% fetal bovine serum and 1% astrocyte growth supplement (ScienCell). The MGMT-negative primary cell line (SG2) derived from GBM surgical specimens was maintained in primary serum-free culture and was cultured in DMEM supplemented with 10% FBS.

2.2. Isolation of exosomes

Cultured cells grown to 90% confluence were washed with PBS and then were incubated with complete medium containing exosome-free FBS (System Biosciences, USA) for 48 h. The exosomes were isolated from the conditioned medium by differential centrifugation using an Optima L-100 XP Ultracentrifuge (Beckman Coulter, USA). In brief, conditioned medium was centrifuged at 300 g for 10 min and then at 2000 g for 20 min at 4 °C to remove cells, followed by filtration through a 0.22 μm filter to remove cell debris. Next, exosomes were pelleted by ultracentrifugation at 100,000 g for 70 min. The pelleted exosomes were resuspended in PBS and were stored at −80 °C [14,26]. The size and concentration of the exosomes were quantified by qNano (Izon Science, New Zealand) and the exosomes were ready for RNA/protein extraction or cell treatment.

2.3. Transmission electron microscopy

Exosomes were mixed with an equal volume of 4% paraformaldehyde for electron microscopy analysis. Next, samples were deposited onto Formvar-carbon-coated electron microscopy grids, washed with PBS and further fixed with 1% glutaraldehyde for 5 min. The samples were contrasted first in a solution of uranyl oxalate, pH 7 for 5 min and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose for 10 min. Finally, the samples were observed using a JEM 1010 transmission electron microscope at 80 kV.

2.4. Exosome labeling and analysis

The protein content of exosomes was measured using the MicroBCA protein assay (Thermo Scientific, USA). The positive exosomal markers CD63 and CD81, and negative marker Calnexin were tested by Western blotting. For exosome uptake experiments, the exosomes were labeled with the DiO (green) or DiI (red) Cell-Labeling Solution (Thermo Scientific, USA) was used according to the manufacturer's protocol.

2.5. Non-contact cell co-culture system

Different sizes of Transwell including Transwell-6, 24, 96, and 100 mm system were used for different analyses. Glioma cells were plated in 0.4 μm porous Transwell inserts (Corning Inc., USA) suspended over normal astrocyte cells plated at different ratio and co-cultured for different days. Mixing medium with DMEM and astrocyte medium was used for co-culture.

2.6. Tumor xenograft models and therapeutic regimens

Animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health (documentation 55, 2001) and were performed in accordance with the approved guidelines and experimental protocol of Nanjing Medical University. For subcutaneous xenograft studies, glioma cells (1×10^7) were inoculated into the bilateral thighs of 6-week-old NOD/SCID mice which were purchased from Cancer Institute of the Chinese Academy of Medical Science. When the subcutaneous tumors reached a volume of 50 mm^3 (10 days after subcutaneous injections of tumor cells), ten mice bearing a similar volume of bilateral tumors were randomly divided into two groups (n = 5, DMSO-treated and the TMZ-treated group). All subcutaneous tumors on the left were intratumorally injected with 50 μg of RAS-EXOs dissolved in PBS (1/5 volume of tumor) every five days for 20 days. The right tumors were injected with the same volume of PBS. Meanwhile, each tumor-bearing mouse received five intraperitoneal injections of DMSO or TMZ for 5 days per week for 2 weeks. Finally, the mice were sacrificed by cervical dislocation under general anesthesia with chloral hydrate (5%, 100 μL/10 g) 35 days after the subcutaneous injections of tumor cells. The tumor volume (V) was monitored by measuring the tumor length (L) and width (W) with calipers and then calculated with the formula $(L \times W^2) \times 0.5$.

2.7. Statistical analysis

All in vitro experiments were performed in triplicate with means and standard error of the mean or standard deviation subjected to Student's t-test for pairwise comparison or ANOVA for multivariate analysis. Kaplan-Meier survival analysis was performed using Graphpad Prism 5 software. A significance level set at $P < 0.05$ was considered significant for all the tests (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).