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Cathepsin L silencing increases As_2O_3 toxicity in malignantly transformed pilocytic astrocytoma MPA58 cells by activating caspases 3/7

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ABSTRACT

Low-grade, pilocytic astrocytomas are treated by resection, but additional therapy is necessary for those tumors with anaplastic features. Arsenic trioxide (As_2O_3) is emerging as an effective chemotherapeutic agent for treatment of malignant glioblastoma multiforme, where Cathepsin L silencing enables lower, less harmful As_2O_3 concentrations to achieve the desired cytotoxic effect. Here, we evaluated the effects of As_2O_3 combined with stable Cathepsin L shRNA silencing on cell viability/metabolic activity, and apoptosis in primary cultures of recurrent malignantly transformed pilocytic astrocytoma (MPA). These cells expressed high Cathepsin L levels, and when grown as monolayers and spheroids, they were more resistant to As_2O_3 than the U87MG glioblastoma cell line. Caspases 3/7 activity in MPA58 spheroids was not significantly affected by As_2O_3, possibly due to higher chemoresistance of primary biopsy tissue of less malignant astrocytoma versus the malignant U87MG cell line. However, As_2O_3 treatment was cytotoxic to MPA spheroids after silencing of Cathepsin L expression. While Cathepsin L silencing only slightly decreased the live/dead cell ratio in As_2O_3-treated MPA-si spheroids under our experimental conditions, there was an increase in As_2O_3-mediated apoptosis in MPA-si spheroids, as indicated by elevated caspases 3/7 activity. Therefore, Cathepsin L silencing by gene manipulation can be applied when a more aggressive approach is needed in treatment of pilocytic astrocytomas with anaplastic features.

1. Introduction

Pilocytic astrocytomas (PAs) are benign brain tumors (World Health Organization [WHO] grade I) that occur more frequently in the pediatric population [1–4]. Although malignant transformation of PAs is not common, there have been several reports of patients with PAs with anaplastic features [4–8], which have been classified as grade II [6] or grade III glioma by the WHO [5,6]. However, PAs with malignant features like necrosis and a high mitotic rate are not an official astrocytoma subtype [4,6]. Despite this, in order to predict disease outcome and choose more aggressive treatments, it is very important to distinguish between PAs and PAs with anaplastic features [5]. Moreover, although surgical resection of PAs prolongs survival rates [3], some tumors recur with malignant transformation [2], in particular, those treated with radiation therapy [3,8,9]. PAs with anaplastic features demand more harsh radiotherapy and chemotherapy treatments [4,6], while new therapeutic modalities are also needed.

Arsenic trioxide (As_2O_3) has been used for treatment of acute

Abbreviations: AMC, 7-amimo-4-methylcoumarin; APL, acute promyelocytic leukemia; As_2O_3, arsenic trioxide; Bax, BCL2-associated X protein; Bcl2, B-cell CLL/lymphoma2; CatL, Cathepsin L; DMEM, Dulbecco's modified Eagle's growth medium; GBM, glioblastoma multiforme; MPA58, malignantly transformed pilocytic astrocytoma 58 cells; MPA58sc, MPA58 cells transduced with lentiviruses encoding scrambled shRNA expression vector; MPA58si, MPA58 cells transduced with lentiviruses encoding shRNA expression vector for stable silencing of Cathepsin L; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; p53, tumor protein 53; PA, pilocytic astrocytoma; PML protein, promyelocytic leukemia protein; shRNA, short hairpin RNA; siRNA, small interfering RNA; STS, staurosporine; TNFα, tumor necrosis factor alpha; WHO, World Health Organization

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promyelocytic leukemia (APL) [10,11], and more recently for treatment of gliomas. In a phase I study in children with anaplastic astrocytoma, anti-tumor activity of As_2O_3 was observed when used in combination with radiation therapy [12]. As_2O_3 has also been used in combination with radiation and temozolomide in patients with anaplastic astrocytoma and anaplastic oligoastrocytoma [13]. At a cellular level, As_2O_3 promotes apoptosis of tumor cells [14–18], where it affects the intrinsic apoptotic pathway through mitochondrial release of cytochrome C and activation of executioner caspases 3/7 [15,16,19–21]. Pucer and co-workers [15] showed significant enhancement of apoptosis induced by As_2O_3 with simultaneous Cathepsin L (CatL) inactivation, which was provided by using the inhibitor Clik148 and by temporary siRNA silencing. CatL is a cysteine endopeptidase and its expression is increased in various tumor types, including astrocytoma and glioblastoma (GBM) [22,23]. As well as CatL, other cysteine cathepsins are highly up-regulated in GBM [24–26], including CatK [27], CatB, and CatS, and they have functional roles in tumor progression [28,29]. For example, whereas we and others have demonstrated that CatB is associated with GBM cell invasion in experimental studies [25,30] and bad prognosis in clinical studies [23], CatL expression was not associated with increased invasiveness of GBM cells using more selective inhibitors of CatL [25], and it did not have a prognostic impact in GBM [23]. In general, CatL is associated with tumor growth and progression [31], while its most intriguing role is in drug resistance [32,33] and apoptosis, as demonstrated in GBM cells by Kenig and co-workers [34].

The synergistic effects of As_2O_3 and CatL inactivation were first shown in the GBM U87MG cell line when grown as a monolayer [15], and then later in U87MG spheroids with stably silenced CatL [18]. In the present study, we tested the joint effects of As_2O_3 exposure and CatL silencing on primary glioma monolayers and spheroids obtained from a recurrent malignantly transformed pilocytic astrocytoma (MPA), to investigate whether these MPA cells respond in a manner similar to the GBM U87MG cells. The enhanced effect of CatL inactivation on As_2O_3 cytotoxicity might be exploited in therapy to reduce the doses of As_2O_3 needed, through simultaneous down-regulation of the highly expressed CatL in tumors. This combination would enhance the treatment cytotoxicity for the cancer cells, while reducing the unwanted side effects due to the lower dose of As_2O_3 used, as As_2O_3 can cause defects in liver functioning and homeostasis of other organs [35].

2. Material and methods

2.1. Cell culture

2.1.1. Establishment of Glioma cultures

Samples of brain tumors were obtained through collaboration with Haukeland Hospital in Bergen, Norway. Tumor diagnoses were made using established histopathology procedures, in the Department of Pathology, University of Bergen, Norway, in accordance with the Regional Committee for Medical and Health Ethics, Norway (REK 013.09). Tumor tissue samples (GBM7, GBM15, GBM17, GBM18, GBM19, GBM20, GBM36, GBM54, MPA58) were transferred into ice-cold Dulbecco's modified Eagle's growth medium (DMEM; Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (A15-104), 2 mM L-glutamine, 1% penicillin/streptomycin (P11-010; all PAA Laboratories GmbH, Austria), 1% non-essential amino acid (M7145; Sigma-Aldrich, Germany), 1 mM sodium pyruvate (Life Technologies, USA), 5 μg/mL plasmocin (Invitrogen, USA) and 10 μg/mL fungin (Invitrogen, USA). These were then mechanically disrupted into 0.5-mm-size pieces, and cultivated on plates that were pre-coated with 0.75% agar (BD Difco, USA) in DMEM media [36]. After 4–19 days of incubation at 5% CO_2 and 37 °C, organotypic tumor spheroids were formed.

2.1.2. Preparation of monolayers and similar-sized spheroids

Neural tissue dissociation kit (Papain; Miltenyi Biotec, USA) was used for enzymatic degradation of the organotypic spheroids, according to the manufacturer instructions. The spheroids were washed in Hanks' balanced salt solution without Ca and Mg (Gibco Products, Invitrogen, USA), incubated with the different enzyme mixtures, and mechanically dissociated using a Pasteur pipette. The cell suspensions obtained were strained through 40-μm-mesh nylon cell strainer (BD Falcon, Bedford MA, USA), washed twice with Hanks' balanced salt solution with Ca and Mg (Gibco Products, Invitrogen, USA), and re-suspended in the above-described culture medium. Part of each cell suspension was seeded on tissue-culture-treated plates (Corning, USA) to be grown as a monolayer in the same medium as the organotypic spheroids. The established primary monolayers were used to prepare similar-sized spheroids, with diameters of 450 μm to 550 μm. Here, 5×10^3 cells per well were seeded in non–cell-culture-treated U-bottomed 96-well plates (BD Falcon, USA) in DMEM growth medium, as described above, with 10% methylcellulose added (Sigma-Aldrich, Germany), and centrifuged at $850\times g$ for 1.5 h. The spheroids formed after 48 h of cultivation in 5% CO_2 at 37 °C were used for experiments.

2.1.3. Spontaneous formation of undefined MPA58 spheroids from cell suspensions

As single cancer cells are capable to form three-dimensional spheroids [37], MPA58 cells grown as monolayers were trypsinized, and 1.3×10^6 cells were transferred to a 75-cm^2 plate coated with 0.75% agar in DMEM, to allow spheroid formation. After 4 days, globular three-dimensional structures, called spheroids, were formed and their images were taken.

2.2. Arsenic trioxide treatment

For the stock solution of arsenic trioxide (As_2O_3; Merck, Germany), its powder was dissolved in 1.5 mM NaOH and diluted to 10 mM with MilliQ water. Arsenite ion (As^{3+}) concentration and the absence of oxidized arsenate ion (As^{5+}) were confirmed as described by Pucer and co-workers [15]. Before each treatment, the As_2O_3 stock solution was diluted with growth medium to the required final concentrations (i.e., 2, 5, 10, 20 μM) and used for 24 h or 72 h treatments of cells and spheroids.

2.3. Cathepsin L mRNA and protein expression

RNA was isolated from MPA58 cells using the TRIzol reagent (Life Technologies, USA) according to the manufacturer instructions, and quantified by NanoDrop ND-1000 (Thermo Scientific, USA). cDNA was generated from 500 ng RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), according to the manufacturer protocol. The expression of CatL was quantified by qRT-PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA), using the cDNA obtained, with TaqMan Universal PCR Master Mix (Applied Biosystems, USA), and the TaqMan gene expression assay for Cathepsin L1 (CTSL1 Hs00377632_m1; Applied Biosystems, USA). Amplification of glyceraldehyde 3-phosphate dehydrogenase (Pre-Developed TaqMan Assay Reagent No. 4310884E; Applied Biosystems, USA) was used as the endogenous control. The data were analyzed by the comparative Ct Method (ΔΔCt algorithm) using the SDS 2.3 software. Independent experiments were performed in duplicate, and repeated three times.

Western blotting was used to detect CatL expression at the protein level. Control and transduced MPA58 cells were suspended in homogenization buffer (50 mM Tris-HCl, pH 6.9, 0.05% Brij 35, 0.5 mM dithioerythritol, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin A), lysed through three freeze/ thaw cycles, sonicated, and centrifuged at $10{,}000\times g$ for 20 min at 4 °C. The concentrations of the isolated proteins were determined using the Bradford assay (Bio-