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Knockdown of *AKT3* and *PI3KCA* by RNA interference changes the expression of the genes that are related to apoptosis and autophagy in T98G glioblastoma multiforme cells

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ABSTRACT

Introduction: Glioblastoma multiforme (GBM) is the most malignant and invasive human brain tumor and it is characterized by a poor prognosis and short survival time. The PI3K/AKT/PTEN signaling pathway plays a crucial role in GBM development and it is connected with the regulation of apoptosis and autophagy. Akt is involved in various aspects of cancer cell biology such as cell survival, in addition to both apoptosis and autophagy.

The current study was undertaken to examine the effect of the siRNAs that target AKT3 and PI3KCA genes on the apoptosis and autophagy of T98G cells.

Methods: T98G cells were transfected with AKT3 and/or PI3KCA siRNAs. Alterations in the mRNA expression of apoptosis- and autophagy-related genes were analyzed using QRT-PCR. LC3IIA protein-positive cells were identified using flow cytometry with specific antibodies.

Results: Our findings demonstrate for the first time that the siRNAs that target AKT3 and PI3KCA change the expression of the genes that are related to apoptosis and autophagy and change the expression of the LC3IIA protein in T98G cells.

Conclusions: Thus, there is a high probability that the knockdown of these genes induces apoptosis and autophagy in T98G cells, but further studies are necessary in order to clarify and check whether autophagy induction is a positive phenomenon for the treatment of GBM.

Introduction

Glioblastoma multiforme

Glioblastoma multiforme (GBM; WHO grade IV) is the most common and lethal primary brain tumor in adults. GBM is characterized by extensive brain tissue migration and infiltration [1]. These unfavorable features result in poor patient survival due to an insufficient surgical resection. Inefficient adjuvant treatment of the tumor's residual infiltrative component is mediated by the high resistance of glioblastoma to radiation and chemotherapy, mainly through the overexpression of the PI3K/Akt pathway [2,3], which has an impact on cellular proliferation, growth, survival, motility, differentiation, metabolism, protein synthesis as well as regulating tumor angiogenesis and invasiveness [4]. The highly invasive and therapy-resistant character of GBM results in the shortest survival time of all cancers (the median survival <15 months for patients with newly diagnosed cancer regardless of their treatment methods) [5]. Current treatment strategies for GBM using surgery, chemotherapy and/or radiotherapy are ineffective, but have triggered a great deal of research effort worldwide for new treatment modalities that can be applicable to this cancer.

PI3K/Akt pathway

PI3Ks, which is a family of phosphoinositide 3-kinases, has been divided into three classes (I – described below; II – consists of single catalytic subunits: isoforms PI3KC2α, PI3KC2β and PI3KC2γ) and III – involves a single catalytic subunit Vps34). Class I consists of two subclasses – class IA and IB, respectively. Class IA contains heterodimers that are composed of a p110 catalytic subunit and a

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p85 regulatory subunit. The p110 subunit has three isoforms (p110α, p110β, p110γ), which are involved in the regulation of the proliferation, survival, migration, degranulation and vesicular trafficking. It is suggested that only the gene encoding p110α subunit plays a crucial role in tumorigenesis [5,6]. Further, it is reported that the knockdown of *PI3KCA* interferes with PI3K/Akt signaling [7]. Zhou et al. [8] indicate that the knockdown of *PI3KCA* by siRNA may result in a decreased catalytic activity of PI3K. The *PI3KCA* gene was found to be amplified and overexpressed in several types of human cancers and the activating point mutations as well as gene amplification of *PI3KCA* have been reported in human brain tumors including GBM [5]. Mutations or amplification of the *PI3KCA* gene have been reported to constitutively increase PI3K activity in cancer cells. Akt is a major downstream effector of PI3K and impinges on numerous cellular processes as has previously been written. Akt activation has been observed in approximately 80% of human GBM [9], which is well correlated with the fact that RTKs/PI3K/Akt signaling is altered in 88% of GBM [5]. There are three closely related isoforms of Akt (pathologically amplified in human cancers) [10]: Akt1, Akt2 and Akt3. Expression of the last one is more restricted to the neuronal tissue than its other isoforms [11]. The Akt2 and Akt3 are overexpressed in glioma cells and play a pivotal role in the malignancy of gliomas [4]. Akt is involved in a various aspects of cancer cell biology such as cell survival, in addition to both apoptosis and autophagy [12].

Apoptosis and autophagy regulation via *the PI3K/Akt pathway*

Activation of the PI3K/Akt pathway is connected to the phosphorylation of numerous effector proteins and is also related to apoptosis and autophagy. The activation of this pathway leads to the suppression of apoptotic cell death through an Akt direct and indirect manner [13]. Phosphorylation of the Bcl-2 family proapoptotic proteins such as Bad and Bax leads to their inactivation, degradation or changes in their cellular location. Similarly, the phosphorylation of procaspase 9 inhibits its proteolytic maturation and thus stops the subsequent activation of effector caspases. Akt inhibits apoptosis indirectly through its NF-κB activity, which is mediated by the transcription of antiapoptotic proteins. Activated Akt kinase interferes with mitochondrial outer membrane permeabilization, thereby also suppressing cell death in a Bad-independent manner [14]. Furthermore, Akt kinase is responsible for the inhibition of the FOXO family proteins that regulate the transcription of the Bim and FasL pro-apoptotic proteins.

One of the most important Akt effector proteins is mTOR kinase, which intimately linked to PI3K/Akt signaling and to the regulation of protein synthesis, cell growth and survival [15]. mTOR is a major negative regulator of the autophagy process. Activated PI3K/Akt signaling leads to the inhibition of autophagy through TSC1/2 phosphorylation by Akt. Once the mTORC1 kinase activity is inhibited, autophagosome formation occurs [16].

Therefore, it is very important to discover the exact mechanisms that determine the PI3K/Akt signaling activity and to understand how the inhibition of this pathway influences GBM cell death. The current study was undertaken to examine the effect of the siRNAs that target the *AKT3* and *PI3KCA* genes on the apoptosis- and autophagy-related genes expression of T98G cells.

Methods

Cell cultures

The T98G cell line, which was derived from a 61-year-old male [17] and purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), was cultured in a modified Eagle's minimum Essential Medium (ATCC) that was supplemented with heat-inactivated 10% fetal bovine serum (ATCC) and 10 μg/ml gentamicin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

siRNA transfection

T98G cells were seeded at 1.6×10^4 cells per well in six-well plates and incubated for 24 h. Next, the T98G cell line was transfected with the specific siRNAs that target *AKT3* (without affecting *AKT1* and *AKT2* mRNAs level) or *PI3KCA* mRNA using the FlexiTube siRNA Premix (Qiagen, Italy) according to the manufacturer's protocol. The following target sequences were used: 5′ AACTGTTGGCTTTGGATTAAA 3′ (for *AKT3*) and 5′ CTGAGTCAGTATAAGTATATA 3′ (for *PI3KCA*). Optimum transfection conditions and transfection efficiency were established as has previously been described [18]. AllStars Negative Control siRNA was also tested (Qiagen, Italy) as we previously described [18]. After transfection, but prior to performing assays, the cells were washed with PBS, trypsinized and centrifuged (125 × *g*/5 min) at 4 °C (listed below).

RNA extraction

Total RNA was isolated from cultured cells using the TRIzol reagent (Life Technologies, Inc, Grand Island, NY, USA) according to the manufacturer's protocol. The integrity of total RNA was checked using electrophoresis in 1% agarose gel stained with ethidium bromide. All RNA extracts were treated with *DNAse I* to avoid genomic DNA contamination and assessed qualitatively and quantitatively.

The evaluation of the transcriptional activity of apoptosis and autophagy-related genes by QRT-PCR

QRT-PCR assays were performed using an ABI Prism7700 (Applied Biosystems, Foster City, USA). Real-time fluorescent RT-PCR was performed using a KiCqStart Primers (Sigma Aldrich) and SensiFast SYBR Hi-ROX One-Step Kit (Bioline) according to the manufacturer's protocol under the following conditions: 45 °C for 10 min, 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C and 5 s at 72 °C. RNA for human Tata Binding Protein (TBP) was used as an endogenous control. The copy numbers for each sample were calculated using the C_T-based calibrated standard curve method. Each data point is the mean of triplicate measurements. Twelve replicates were used for each gene analysis.

The evaluation of LC3IIA protein expression using flow cytometry

LC3IIA protein-positive cells were identified using direct labeling with a specific rabbit anti-LC3IIA antibody (isotype IgG_A) and flow cytometry. In order to confirm that the observed effects are autophagy-specific, an inducer (N-hexanoyl-D-sphingosine, 10 μM for 24 h) and an inhibitor (bafilomycin A1, 100 nM for 2 h), were used. After *AKT3* and *PI3KCA* knockdown, cells were harvested, washed twice in PBS and fixed in PBS with 4% paraformaldehyde. Then, cells were washed twice in PBS with 1% BSA, permeabilized with 0.1% saponin/1% BSA/PBS for 45 min and incubated overnight at 4 °C with 10 μg/ml of an anti-LC3IIA polyclonal antibody (antibody concentration: 1 μg/μl) conjugated with FITC (Bioss) in 1% BSA/PBS. An isotype-matched monoclonal antibody (isotype control) was used to determine non-specific binding. Subsequently, cells were washed and analyzed using a FACSAria II (BD Biosciences) equipped with Diva Software. Four biological replicates were used in the flow cytometry analysis for the LC3IIA protein measurement. When autophagy is activated, the LC3-I protein that is localized in the cytoplasm is cleaved, lipidated

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