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ORIGINAL ARTICLE

Downregulation of miR-195 via cyclosporin A in human glioblastoma cells

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Summary

Purpose: *Cyclosporin A (CsA) is a potent immunosuppressive agent. MicroRNAs (miRs) which post-transcriptionally regulate gene expression are non-coding RNAs. The aim of this study was to investigate the effects of CsA on 88 miRs expression changes in glioma cells (U-87 MG).*

Methods: *CsA was used in U-87 MG glioma cells in doses of 10, 30 and 60 μM. Cytotoxic assays and determination of IC_{50} dose of CsA were performed. Relative quantification of 88 miRs was performed by real time RT-PCR. The fold changes of miRs determined and alterations in the miR expressions were compared with CsA-treated and CsA- free U-87 MG glioma cells.*

Results: *In U-87 MG cells treated with CsA, the IC_{50} dose was 10 μM. Seventeen of 88 human miRs were downregulated compared to the untreated control group by using miRs array. It was found that the expression levels of several miRs, in particular miR-195, was significantly decreased in CsA-treated U-87 MG cells.*

Conclusion: *This study revealed a significant role of miR-195 in the molecular pathology of glioma cells which can also implicate potential application of miR-195 in cancer therapy. Rather than downregulation of miR-195 alone to exhibit cytotoxicity, treatment with CsA could be more effective especially on temozolomide-resistant cells.*

Key words: *cyclosporin A, glioma, miR-195, U-87 MG cells*

Introduction

Glioblastoma (GBM) is the most common primary malignant neoplasm of the central nervous system in adults and the patients usually have a short survival time of less than a year [1]. The molecular consequences of the genetic changes that underlie the malignant phenotype of GBM have been widely studied and these studies have contributed to the development of new diagnostic and therapeutic methods [2].

The very powerful immunosuppressive agent CsA lacks myelotoxicity which makes it distinctive among other non-steroidal drugs given for immunosuppression [3]. Along with its immunosuppressive activity, CsA displays also neuroprotective effects, like reducing cell death and cellular changes at prolonged seizure activity, traumatic brain injury and stroke. By inhibiting essential signaling pathways for tumor proliferation and invasiveness, CsA affects the growth and survival of human GBM cells and prevents significantly their growth *in vivo* [4,5].

miRs are a class of non-coding RNAs that are effective in the regulation of gene expression at post-transcriptionally level. They are single-strand, non-coding RNA molecules and consist of 18-25 nucleotides. They are coded by genes which are transcripts from DNA but not translated into proteins. miRs play a role in development,

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differentiation, metabolism, immunity, proliferation and apoptosis [6,7]. In this study, we aimed to investigate the effect of CsA on glioma cell growth via the expression changes of potential 88 miRs in U-87 MG glioma cells.

Methods

Tumor cell line

U-87 MG glioma cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and used as a brain tumor model.

Chemicals and reagents

U87-MG cells were treated with CsA (Sigma Aldrich, St.Louis, MO, USA), at different concentrations ranging from 10 µM to 60 µM for 72 hrs. Cells without any treatment composed the control group.

Cell culture and preparation of cytotoxicity experiments

U-87 MG cells were grown in BIOAMF-1 basal medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10 000 U/ml penicillin, 10 mg/ml streptomycin and 2 mM L-glutamine. The culture was maintained in a standard cell culture incubator at 37°C, humidified 95% air, and 5% CO_2 atmosphere until cells were confluent. Prior to any experiment, $5x10^5$ cells/ml in suspension were aliquoted into flasks for subsequent manipulations.

Cytotoxicity assay

Cytotoxicity assay and determination of IC_{50} dose of CsA in glioma cells were performed by using trypan blue dye exclusion test and XTT assay after 24, 48 and 72 hrs of exposure to CsA as indicated by the manufacturer's instructions. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 620 nM), by a microplate reader (Multiskan FC, Thermo Scientific, Vantaa, Finland). Viability was calculated by using the background-corrected absorbance. In U-87 MG cells treated with CsA, IC_{50} dose was 10 µM.

Isolation of miR

miR was isolated from cells exposed to IC_{50} doses of CsA and the control group. Isolation of miR and cDNA synthesis was performed by using RT^2 qPCR-Grade miR Isolation Kit and RT^2 first Strand Kit (Qiagen, Valencia, CA, USA) respectively according to the manufacturer's instructions.

Relative quantification of miRs

Relative quantitation of 88 miRs (Human Genome RT^2 miR PCR Array, MAH-001, SA Biosciences, Frederick, MD, USA) was measured by Light Cycler 480 Real Time RT-PCR (Roche Applied Science, Indianapolis, IN, USA). miRs expressions were normalized to SNORD48 (Small nucleolar RNA, C/D BOX48), SNORD47, SNORD44 and U6. Fold changes of miRs determined and Log2 transformation were assessed. Alterations in the miRs expressions were analysed in CsA-treated and CsA-free U87-MG glioma cells.

Statistics

The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in miR expression. Data was analyzed with Web-Based RT^2 profiler PCR array data analysis (SA Biosciences, Frederick, MD, USA). A difference more than ±2 fold change expression was accepted as the cut-off value. P values <0.05 were considered statistically significant.

Results

CsA exerted its cytotoxic effect on U-87 MG cells in a time- and dose-dependent manner. A relative reduction in the cell number that evolved in the cell line cultures was observed. Assays were performed to determine U-87 MG cells viability and miR expression showed a significant change in CsA-treated samples. The IC_{50} of CsA was 10 µM by trypan blue dye exclusion test followed

Table 1. miR expression change in U-87 MG cells treated with CsA compared to the control group

miR	*Fold change*
hsa-miR-195	-6.93
hsa-miR-30c	-3.18
hsa-miR-30b	-2.86
hsa-miR-146a	-2.33
hsa-miR-142-5p	-2.18
hsa-miR-142-3p	-2.18
hsa-miR-126	-2.18
hsa-let-7f	-2.18
hsa-miR-9	-2.18
hsa-miR-144	-2.18
hsa-miR-32	-2.18
hsa-miR-140-5p	-2.18
hsa-miR-141	-2.18
hsa-miR-26b	-2.18
hsa-miR-101	-2.18
hsa-miR-374a	-2.18
hsa-miR-302b	-2.18

by a confirmation by XTT assay. In U-87 MG cells treated with CsA (10 μM), 17 of 88 human miRs were significantly downregulated ($p<0.05$) compared to the control group (Table 1). It was found that the expression levels of several miRs, in particular miRNA-195, were significant decreased up to 7-fold in the CsA treated U-87 MG cells.

Discussion

There is an urgent need to develop effective therapeutic strategies leading to improvement of survival of glioblastoma patients. Recently, studies have shown an increasing number of miRs deregulation was a common event in cancer [8,9]. Various experimental approaches have shown that miRs may be considerable therapeutic targets for cancer treatment [10,11]. A study has shown that miRs also help establish the molecular diagnosis, prognosis and treatment of glioblastoma [12].

This study demonstrated that miR-195 may downregulate glioblastoma cells by inhibiting the cell proliferation with CsA treatment. The results of the present study indicated that the expression level of miR-195 may help differentiate patients with glioblastoma with good or bad prognosis. Xu et al. have reported that miR-195 arrests the cell cycle progression by targeting or by repressing genes in other cancer cell lines [13]. The action of CsA on apoptosis shows that CsA can induce the apoptotic process depending on cell type and conditions. CsA induced apoptosis of rat C6 glioma cells [14], and another study on the biological effects of CsA in glioblastoma U251MG cells indicated that cyclosporin has imposed multiple inhibitory actions on glioma cells, such as IL-8 production and proliferation [15].

miR-195 is a highly conserved miR, it exhibits different expression patterns and functions in different types of cancer and it is significantly downregulated in glioblastoma cell lines compared with normal brain tissues [16,17]. Since miR-195 regulates genes like BCL2, CNOT6L, USP15, PAFAH1B1 and ESRRG, it may be considered as a candidate tumor suppressor in glioblastoma cells [18]. The role of deregulation of miR-195 is uncertain in glioblastoma development.

Downregulation of miR-195 may be a common occurrence in tumor development [19] and may promote cell proliferation and tumorigenicity of cancers [13,20,21], whereas opposite reports have shown that downregulation of miR-195 is associated with poor prognosis in adrenocortical carcinomas and suppresses both cell proliferation and invasion in glioblastoma [2,22].

On the other hand, Ujifuku et al. demonstrated that miR-195 showed 3-fold increased expression in acquired temozolomide (TMZ) resistance in GBM cells and knockdown of miR-195 can reverse the TMZ resistance [23]. In the present study, miR-195 was downregulated (-6.93-fold change) with CsA treatment. We can speculate that miR-195 downregulation may be associated with a poorer prognosis in GBM and CsA treatment could be useful to combat the TMZ resistance via decreased of miR-195 expression.

In conclusion, this study may provide important roles of miR-195 in GBM pathogenesis and in the molecular etiology of GBM. miR-195 expression was reduced by CsA treatment and downregulation in human glioma cells suggests that it might act as an oncogene and trigger the formation of GBM. We need to understand all the roles of miR-195 in cancer development and further research to this direction is warranted.

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