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ALTERNATIVE SPLICING OF mRNA TRAIL REGULATES APOPTOSIS IN THE GLIOBLASTOMA MULTIFORME T-98G CELL LINE

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ABSTRACT

Objective: This is an *in vitro* experimental study designed to analyze the role of alternative splicing of mRNA in the apoptotic process of the cancer cells. Here we induced apoptosis in the glioblastoma multiforme (GBM) T-98G cell line to obtain a better understanding in the regulation of mRNA expression of the soluble Tumor Necrosis factor-related Apoptosis-Inducing Ligand (sTRAIL) gene.

Methods: Cells were induced to undergo apoptosis by treatment with rotenone at 10, 20 and 40 µM for 6 h. Dimethylsulphoxide (DMSO) was used to dissolve rotenone and as a negative control. The morphology of the GBM-T98G cells was viewed with an inverted microscope. DNA, RNA and protein extractions were performed to analyse apoptotic DNA fragmentation by a DNA laddering assay, a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for TRAIL mRNA expression and ELISA for caspase-9 protein expression. Electrophoresis was also performed on TRAIL complementary DNA (cDNA) produced from TRAIL qRT-PCR mRNA.

Results: Nucleosomal DNA degradation was confirmed by DNA laddering, whereas the TRAIL melting curve and the cDNA electrophoresis showed a shift in the balance of the TRAIL mRNA isoform to the pro-apoptotic mRNA isoform, in conjunction with a significant increase in expression of TRAIL mRNA and caspase-9 protein.

Conclusion: These findings indicate the regulation of apoptotic events at the level of TRAIL mRNA expression, as indicated by the shift in the balance of mRNA expression of the TRAIL isoform towards the pro-apoptotic isoform.

Keywords: Pro-apoptotic, mRNA isoform, TRAIL, Glioblastoma multiforme

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and invasive malignant subset of brain tumours and is associated with dismal prognosis [1]. The standard first-line treatment for glioblastoma includes surgery, followed by focal fractionated radiotherapy with concomitant and adjuvant administration of the alkylating chemotherapy temozolomide. The addition of temozolomide significantly improves the median 2-and 5 y survival when compared to radiotherapy alone in patients with newly diagnosed glioblastoma. However, regardless of treatment, glioblastoma patients have a poor prognosis, with a median survival of 14.6 mo [1, 2]. As with many cancers, malignant gliomas show resistance to apoptosis—a characteristic that underlies both tumourigenesis and the inherent resistance of cancer cells to radiotherapy and chemotherapy. Therefore, a full understanding of the cell death mechanism and the genetic regulation behind it is obviously of great medical interest.

Apoptosis can be induced by rotenone (C23H22O6), a plant compound derived from species of the Leguminosae family. Rotenone is a lipophilic compound, so it readily crosses the blood-brain barrier. Rotenone also shows neurotoxic properties because it creates oxidative stress by increasing the production of reactive oxygen species (ROS) in the mitochondria [3, 4]. Experimentally, rotenone is used primarily as a mitochondrial complex 1 inhibitor to induce the release of cytochrome C and initiate apoptosis through the intrinsic apoptotic pathway.

Apoptosis is an ordered and highly selective cellular process that occurs in both physiological and pathological conditions. It occurs by two main pathways: the intrinsic apoptotic pathway, which involves mitochondria and the formation of apoptosome multi-protein complexes of cytochrome-C, apaf-1 and caspase-9, and the extrinsic apoptotic pathway, which can be initiated by the association of death receptors with the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). This ligand-receptor association activates the protein initiator caspase-8 to propagate the apoptosis signal by direct cleavage of downstream effector caspases to the executor protein caspase-3. Crosstalk also occurs from the extrinsic apoptotic pathway through Bid, a Bcl-2 family protein with a BH3 domain, which translocates to the mitochondria and triggers the intrinsic apoptotic pathway [5].

The final process of apoptosis is characterized by the occurrence of two stages of DNA degradation. The first is the breakdown of DNA into pieces 50–300K base pairs (bp) in size, followed by internucleosomal cleavage by the enzyme caspase-dependent DNase (CAD) to form multiple oligonucleosomes of 180–200 bp. These cleavages result in the typical DNA ladder pattern observed in agarose electrophoresis gels [5, 6].

In humans, TRAIL is encoded by genes consisting of five exons and four introns on chromosome 3q26.31. TRAIL is produced physiologically as part of the body's defensive immune surveillance mechanism that guards against the presence of cancer cells but disregards normal tissues [7, 8]. TRAIL is a transmembrane protein, and its extracellular domain contains the C terminal that can be cleaved by proteolytic enzymes to generate soluble TRAIL (sTRAIL). TRAIL has five types of receptors, including two death receptors (DR4 and DR5) that have an intracellular death domain that induces apoptosis. The other three receptors are two decoy receptors (DcR1 and DcR2) and one osteoprotegerin receptor (OPG), which does not induce apoptosis but instead acts as a negative regulator of TRAIL.

Unlike normal cells, most malignant cells do not express the decoy receptors and are therefore sensitive to TRAIL. This feature of TRAIL, combined with its antitumor and anti-metastatic properties, forms the basis of current phase-2 clinical trials using monoclonal antibodies targeting DR4 and DR5 in various cancers, including glioblastoma multiforme. Unfortunately, the results are not yet satisfactory, as drug resistance persists due to immune escape by cells undergoing the transformation into malignancies [9–12]. The TRAIL gene is known to be tightly regulated through genetic and epigenetic mechanisms, including the expression of the TRAIL

isoforms α, β, γ and δ, which are functionally different. The full-length TRAIL α has three splice variants: the one that lacks exon 3 is TRAIL β and the one that lacks exons 2 and 3 other is TRAIL γ. The TRAIL δ variant lacks exons 3 and 4. The loss of exon 3 causes a frameshift mutation that results in ectopic expression of the extracellular domain and the loss of the pro-apoptotic functionality of TRAIL [8, 13].

MATERIALS AND METHODS

Cell culture and rotenone treatment

The human glioblastoma multiforme T-98G cell line was grown as a monolayer culture in Dulbecco's Modified Eagle's Medium-high glucose (Gibco®) with 10% foetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin and 1% amphotericin-B at 37 °C in 5% CO2 in air. Rotenone (Sigma Aldrich USA) was dissolved in dimethylsulphoxide (DMSO) and used to induce apoptosis at a dosages of 10, 20 and 40 µM; cells treated with DMSO served as a negative control. After a 6 h incubation, the cells were observed with an inverted microscope at 200× optical magnification. The cell were then collected and lysed for further analysis.

Isolation of DNA, RNA and protein

Tripure Isolation Kit® (Roche) was used to isolate the DNA, RNA and protein from the sample. The procedures of isolation followed the protocols provided in the kit. A bovine serum albumin (BSA) standard protein curve was prepared for the determination of total protein concentrations [14].

DNA ladder assay

Nucleosomal DNA degradation was identified based on the formation of a DNA ladder. The Apoptotic DNA Ladder Kit (Abcam®) was used to detect apoptosis of GBM T-98G cells in control and treated samples by following the instructions contained in the kit.

Caspase-9 protein expression assay

An indirect sandwich ELISA method was used to determine the caspase-9 concentration using a Human Caspase-9 Platinum ELISA Kit (eBioscience®). A caspase-9 standard protein curve was made determination of caspase-9 concentration.

Soluble trail (sTRAIL) gene expression

Complementary DNA for sTRAIL analysis was synthesised from 200ng of total RNA by a quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using the One-step qRT-PCR Kit (KAPA™SYBR®FAST). The melting curve and relative expression of sTRAIL were evaluated by qRT-PCR using specific oligonucleotide primers: 5'-TGCGTGCTGATCGTGATCTT-3' (forward primer) and 5'-TTGGAGTCTTTCTAACGAGCTG-3' (reverse primer). The primer was design based on accession number NM 001190942.1 obtained from the RefSeq NCBI Gene Bank. A non-template control was performed for each experiment, establishing the absence of genomic contamination in the sample. The 18S rRNA was used as a reference gene (house-keeping gene) using primers 5'-AAACGGCTACCACATCCAAG-3' (forward) and 5'-CCTCCAATGGATCCTCGTTA-3' (reverse). Each experiment was run in triplicate. The results of cDNA amplification were represented by Ct values calculated using the Livak method as relative mRNA expression (compared with the control) [15].

Data analysis using the $2^{-\Delta\Delta Ct}$ method

The corresponding cDNA synthesized from each sample was subjected to qRT-PCR. The data were analyzed using the equation described by Livak [15] as follows:

Ratio of target gene expression = $2^{-\Delta\Delta Ct}$

Where the threshold cycle value (Ct) indicates the fractional number at which the amount of amplified target reaches a fixed threshold. The ΔCt was the average Ct of the target gene minus the average Ct of the reference gene, and the ΔΔCt was the average ΔCt of the treated cells minus the average ΔCt of the untreated cells.

Electrophoresis of cDNA sTRAIL

The amplified cDNA fragments produced from qRT-PCR of sTRAIL mRNA were visualised by gel electrophoresis.

Statistical analysis

Significant differences were statistically analysed using a nonparametric Mann-Whitney test. Differences of $P<0.05$ were considered statistically significant.

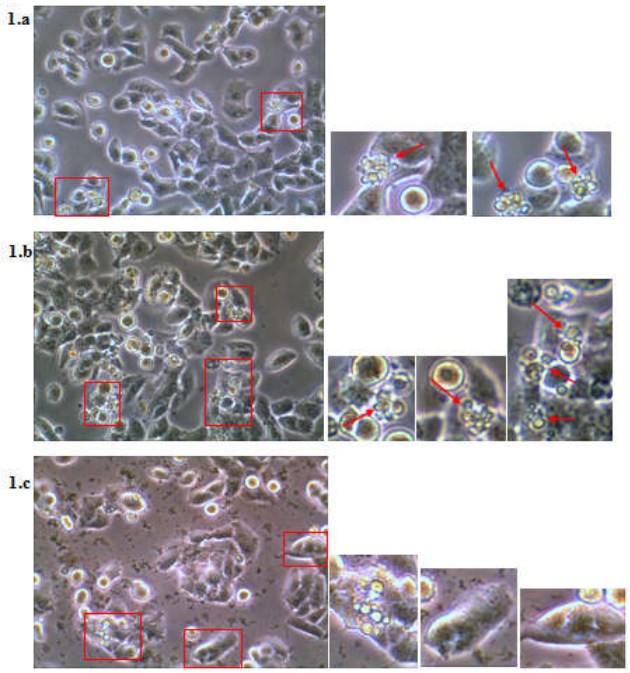

Fig. 1: (a) T-98G cell culture treated with 10 µM rotenone shows membrane budding changes (insert, red arrows); (b) T-98G cell culture treated with 20 µM rotenone shows more membrane budding changes. (insert, red arrows); (c) T-98G cell line treated with 40µM rotenone shows alterations in cell shape accompanied by cell membrane budding (insert)

RESULTS

Observation of the cell morphology showed membrane budding changes in glioblastoma T-98G cells treated with 10 µM rotenone (fig. 1a). Membrane budding changes and cell breakdown were observed in cells treated with 20 µM rotenone, but the cell membranes remained intact (fig. 1b). Cells treated with 40 µM rotenone were swollen, indicating that the cell membranes were no longer intact; these hydropic degeneration picture leads to the process of necrotic degradation (fig. 1c).

Nucleosomal DNA degradation in the form of DNA laddering appeared clearly in the samples treated with 10 and 20µM rotenone. The ladder began to diminish in samples treated with 40 µM rotenone (fig. 2). The control samples treated with DMSO alone were provided with a solvent volume equivalent to that administered the R40 treatment.

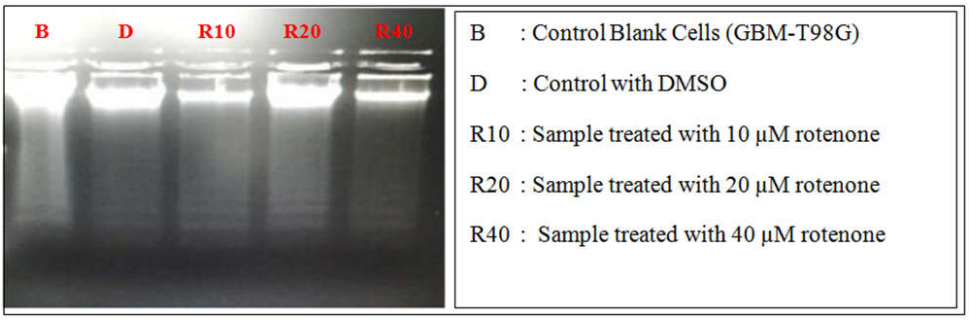

Fig. 2: The DNA ladder indicating internucleosomal DNA fragmentation due to apoptosis

The analysis of caspase-9 protein revealed increased expression in the cells treated with 20 µM rotenone (fig. 3). This result was consistent with the expression of sTRAIL mRNA, which also showed a significant increase in response to 20 µM rotenone (fig. 4).

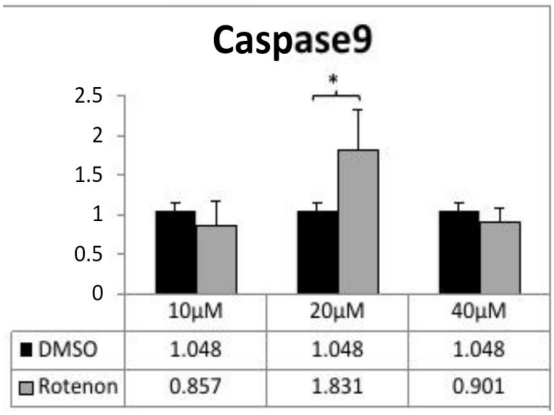

Fig. 3: Relative expression of caspase-9 in T98G cells treated with rotenone or DMSO alone. Treatment with 20 µM rotenone increased caspase-9 expression. * p=0.05

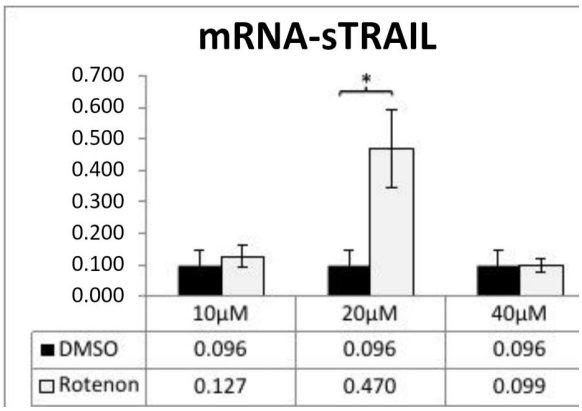

Fig. 4: Relative expression of mRNA-TRAIL in T98G cells treated with rotenone or DMSO alone. The increase in TRAIL expression in 20 µM treated cells was statistically significant. * p<0.05

The sTRAIL melting curve analysis showed two similar melting wave peaks in the control sample. This changed to a single melting wave peak in the rotenone-treated samples (fig. 5a, 5b and 5c).

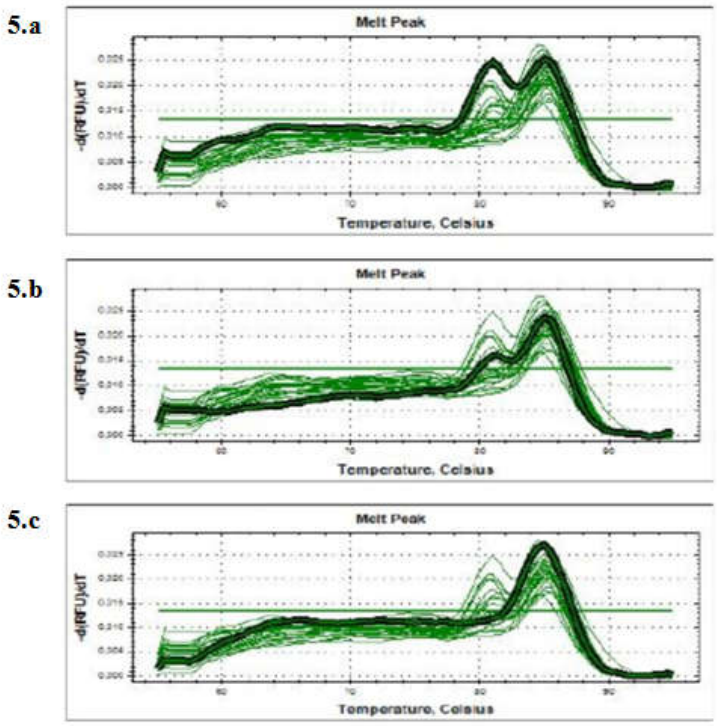

Fig. 5: (a) Melting curve with two peaks for the qRT-PCR of the control sample (DMSO alone); (b) Shifting of the melting curve peak for the qRT-PCR of the sample treated with 10 µM rotenone; (c) Melting curve showing a single peak for the qRT-PCR of the sample treated with 20 µM rotenone

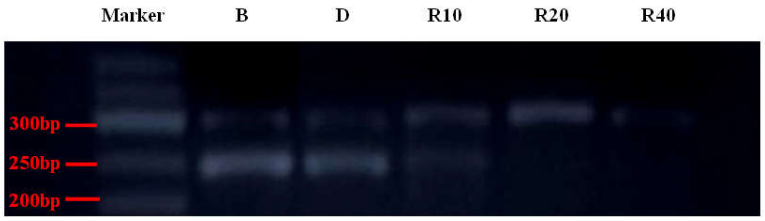

Fig. 6: Two isoforms of TRAIL, with the short band dominant in the cell control treatment (B) and sample treated with DMSO alone (D). The sample treated with 10 µM rotenone (R10) shows a shift towards the longer isoform. The band for the longer isoform becomes more intense in the sample treated with 20 µM rotenone (R20) and the band intensity of the shorter isoform decreases. The long isoform band also begins to disappear in the sample treated with 40 µM rotenone (R40). This phenomenon revealed a transition of expression from the anti-apoptotic isoform to the pro-apoptotic isoform, as well as the end of the apoptotic process and its replacement with necrosis and a loss of gene expression due to DNA degradation

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DISCUSSION

One form of gene regulation is the alternative splicing that occurs during the pre-mRNA maturation process. This splicing process involves the addition or removal of specific exons of a gene so that the mature mRNA translocated to the ribosome carries different instructions for protein synthesis. The isoform proteins produced through alternative splicing play an essential role in several physiological processes involved in cell development, including the regulation of apoptotic mechanisms. Cells undergoing malignant transformation are known to have the ability to interfere with this splicing process, thereby affecting the cell's ability to undergo apoptosis [16].

As one of the natural cytokines produced by immune cells such as T cells and natural killer (NK) cells to induce apoptosis in immune surveillance mechanisms, the anti-tumor potential of TRAIL is undoubtedly. However, phase-2 clinical studies based on the use of recombinant human TRAIL or their agonistic monoclonal antibodies against DR4 and DR5 show less encouraging results even though these death receptors are overexpressed by cancer cells [17]. One of the basics of resistance is the ability of cancer cells to regulate gene expression by alternative splicing of pre-mRNA. The term "better to die than be wrong" has been rearranged in cancer cells by the expression of isoform proteins which support anti-apoptotic

properties. As shown in this study, GBM cells predominantly express the non-apoptotic isoforms of sTRAIL. This regulatory mechanism can be changed by rotenone, which shifts the regulation of sTRAIL gene expression towards the pro-apoptotic isoform.

CONCLUSION

This study demonstrated the beneficial role of rotenone in the process of apoptosis, specifically as a regulator of sTRAIL gene expression at the level of mRNA. This finding underlines the importance of understanding about the regulation of genetic expression in various cases of resistance to cancer targeted therapy. Going forward, the use of specific gene expression regulatory substances that work synergistically with anti-cancer drugs can be an option for therapeutic modalities in cancer.

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Nil

AUTHORS CONTRIBUTIONS

All the author have contributed equally.

CONFLICT OF INTERESTS

The authors declare there is no Conflict of Interest.

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