SUPPRESSION OF MANGANESE SUPEROXIDE DISMUTASE ACTIVITY IN ROTENONE-TREATED HUMAN GLIOBLASTOMA T98G CELLS REDUCES CELL VIABILITY

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INTRODUCTION
Glioma is the most common human primary brain tumor which arises from glial cells [1]. Nowadays, the use of conventional treatments such as chemotherapy and radiation does not significantly enhance the life expectancy of glioma patients, particularly those with high-grade malignant (the WHO Grade IV) multiforme glioblastoma (GBM) patients, which is highly resistant to therapy [2]. Combinatorial treatment strategy has been reported to have improved the chemotherapeutic delivery to tumor cells in the brain [3,4]. Nevertheless, accumulating evidence suggests that dysregulation of cell cycle and apoptosis could lead to radiotherapy resistance [5]. A plausible mechanism for this resistance might involve high antioxidant status in tumor cells which could affect cell survival in response to radiation-induced oxidative stress [6].

Manganese superoxide dismutase (MnSOD) is a major cellular antioxidant located in the mitochondrial matrix. This enzyme catalyzes the reactive superoxide anion into hydrogen peroxide which will be later eliminated by catalase or peroxidase. The previous studies have reported that the upregulation of MnSOD expression could inhibit the phenotype of various cancer cells, suggesting that MnSOD is a tumor suppressor [7-10]. However, this assumption is still controversial, since it has been demonstrated that MnSOD was overexpressed in several human cancers including GBM [11]. Furthermore, MnSOD has been reported to have an important role on tumor cell growth and proliferation in ovarian cancer through regulation of superoxide level [12]. Our recent study has confirmed that MnSOD mRNA expression and specific activity in human glioma cells isolated from clinical specimens were higher than those in normal brain cells. In addition, the oxidative stress biomarkers, i.e., malondialdehyde, carbonyl compounds and 8-OHdG, were notably enhanced [8]. Interestingly, when these cells were categorized based on tumor grade, we found that the high-grade malignant glioma cells expressed MnSOD mRNA at higher levels compared to the low-grade but had lower specific activity.

The differential between MnSOD mRNA level and specific activity in high-grade glioma prompted a line of inquiry to investigate whether this was associated with reactive oxygen species (ROS) levels in these cells. Therefore, the aim of this study was to analyze the effect of rotenone-induced ROS in human GBM on the modulation of MnSOD expression and its association with cell viability. High-dose rotenone was applied to human GBM T98G cells to induce overproduction of intracellular ROS, which is an expected consequence of radiotherapy. Rotenone is an inhibitor of mitochondrial complex I electron transport chain, which increases the mitochondrial ROS generation, particularly superoxide radicals, and leads to cytotoxicity [13-15]. It has also been reported that this toxin could be used as an anticancer agent [15]. Finally, this study is also projected to elaborate on the role of MnSOD in high malignant GBM patients who are resistant to radiotherapy. The outcome of this study has the potential for improving the management of GBM.

MATERIAL AND METHODS

Cell culture
The human GBM cell line T98G (kindly provided by Prof. Alexander Brehm from Institut fuer Molekularbiologie und Tumorforschung Philipps Universitaet Marburg, Germany; ATCC No. CRL-1690™) was maintained in high glucose DMEM containing 10% of heat-inactivated fetal bovine serum, 3.7 g/L of sodium bicarbonate, 1% Streptomycin - Penicillin, and 1% amphotericin B at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were subcultured with 0.25% trypsin and 1% ethylenediaminetetraacetic acid (EDTA) whenever the culture reached confluence. A subcultivation
ratio was 1.2–1.5. Cells were utilized for analysis within 15 passages since it has been demonstrated that antioxidant enzyme levels in tumor cells subcultured up to 50 passages did not change [8].

Rotenone treatment
Rotenone powder (Sigma Aldrich, USA) was first dissolved in dimethyl sulfoxide (DMSO) to obtain 10 mM stock solution. One day before rotenone treatment, 5 X 10⁴ T98G cells were plated triplicate in a 24-well plate and grown in the same medium without free serum. Cells were treated with various concentrations of rotenone and incubated for 6 h, as described previously [12]. Negative controls were T98G cells without rotenone or DMSO treatment, as well as T98G cells treated with the same volume of DMSO used to dissolve rotenone. Rotenone treatment was repeated 3 times at different time points. After the treatment, cells were washed with phosphate-buffered saline (PBS), harvested and then centrifuged at 1000 rpm for 5 min. Finally, cells were counted and prepared either for the cell viability or proliferation analysis, as well as for the isolation of total RNA and protein, followed by the analysis of MnSOD mRNA expression, protein level, and specific activity, respectively.

Determination of intracellular ROS level
Intracellular ROS was measured at the level of hydrogen peroxide and superoxide anion. For peroxide level measurement, dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Molecular Probes, USA) was performed based on the intracellular peroxide-dependent oxidation of 2’,7’-DCFH-DA to form the fluorescent compound 2’,7’-dichlorofluorescein DCF as described previously [16]. Dihydroethidium (DHE) (Invitrogen) assay could detect the presence of superoxide anion which enzymatically converts hydroethidine to ethidium. T98G cell pellets containing 2×10⁴ cells were resuspended in PBS containing 20 µM DCFH-DA or 20 µM DHE and incubated for 30 min at 37°C [17,18]. Cells were then rinsed with PBS (2 times) and centrifuged 1000 rpm for 5 min. Cell pellets were resuspended in 3 ml PBS. Fluorescence intensity was measured using the Microplate Fluorometer (Varioskan Flash®, Thermo Scientific, Finland) at 485 nm and 488 nm excitation, as well as 530 nm and 585 nm emission for DCFH-DA and DHE assays, respectively.

Analysis of cell viability
T98G cell viability was determined using MTS assay according to the manufacturer’s protocol (Cell Titer 96Non-Radioactive Cell Proliferation Assay kit®, Promega, USA). Briefly, ten thousand cells after rotenone treatment were washed with PBS and added with 20 µl of MTS/PSM mixture (MTS/PSM=20:1) to each well and incubated for 1-4 h under standard conditions (5% CO₂ at 37°C). The quantity of formazan product was measured using spectrophotometer at 490 nm, and the absorbance was directly proportional to the number of living cells in culture.

Analysis of cell proliferation
T98G cell proliferation was analyzed using the Cell Proliferation ELISA, BrdU kit® (Roche, Germany) according to the manufacturer’s protocol. After rotenone treatment, 2×10⁴ cells in each 24-well plate were labeled with 20 µl/well of BrdU and incubated for 24 h. The reaction product was quantified by measuring the absorbance at 490 nm using the Microplate Reader (Varioskan Flash®, Thermo Scientific, Finland). The developed color and thereby the absorbance values directly correlated to the amount of DNA synthesis and hereby to the number of proliferating cells.

Analysis of cell apoptosis
Cell apoptosis was analyzed using terminal deoxynucleotidyltransferase (TdT) dUTP Nick-End Labeling (TUNEL) assay (In Situ Cell Death Detection kit®, PDI) according to the manufacturer’s protocol. This assay has been designed to detect apoptotic cells that undergo extensive DNA fragmentation during the late stages of apoptosis. Fifty thousand T98G cells were plated in slide chamber one day before rotenone treatment. The counterstaining process was performed using methyl green solution, as described previously [19]. Cells were observed under light microscope (Nikon ECLIPSE 80i). Dark brown cells were identified as apoptotic cells and calculated using Image J cell counter software (NIH).

Analysis of MnSOD mRNA expression using real-time polymerase chain reaction (RT-PCR)
Total RNA was isolated from harvested T98G cell culture using TriPure Isolation kit® (Roche) according to the manufacturer’s protocol. The concentration of total RNA was determined using spectrophotometer at 260 nm wavelength. cDNA of MnSOD was synthesized from 200 ng of total RNA samples and amplified using iScript one-step RT-PCR Kit with SYBR Green® (BioRad), according to the manufacturer’s protocol. 18S rRNA was used as a reference gene. The amplification protocol was performed using primers for MnSOD or 185rRNA gene, as described in our previous report [20]. Aquabidest was used as a non-template control to exclude the false-positive result. The level of mRNA expression in rotenone-treated or DMSO-treated cells was relatively determined using Livak formula and normalized to that in the cells without any treatment as a control.

Analysis of MnSOD protein
Total protein was isolated from harvested T98G cell culture using TriPure Isolation kit® (Roche) according to manufacturer’s protocol. Total protein concentration in each sample was first measured using spectrophotometer at 280 nm wavelength and plotted to the bovine serum albumin standard curve. The human MnSOD standard stock-solution was reconstituted by adding the dilution buffer to obtain MnSOD concentration of 1600, 800, 400, 200, 100, 50, 25, and 0 pg/mL. MnSOD protein levels were determined using spectrophotometer at 450 nm and calculated using the concentration of standards provided in the kit and the total protein concentration in each sample.

Analysis of the MnSOD enzyme-specific activity
MnSOD enzyme activity was measured using xanthine oxidase inhibition assay (RanSOD® kit, Randox), as previously described [19]. To inhibit the Cu/ZnSOD, first, natrium cyanide (5 mM) was added into each sample, and the mixture was incubated for 5 min in room temperature [21]. MnSOD enzyme activity was expressed as percentage (%) inhibition of the samples plotted to the standard curve. The specific activity of MnSOD enzyme was calculated as enzyme activity (in Unit) per mg total protein.

Analysis of transmission electron microscopy (TEM)
The mitochondrial ultrastructure of T98G cells-treated rotenone was obtained by TEM. Cells were firstly fixed in 2.5 % glutaraldehyde containing 3% sucrose in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C, then in 2.5 % glutaraldehyde containing 3% sucrose in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h at 4°C, and finally in 2% osmium tetroxide and 2.5% K₄Fe(CN)₆ in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C [22]. Afterward, samples were dehydrated in graded ethanol for 15 min, embedded in Spur’s resin for 24 h at room temperature and examined under a JEOL 1010 transmission electron microscope.

Statistical analysis
Statistical analysis was performed using Student’s t-test (for comparison between rotenone- and DMSO-treated cells) or Wilcoxon test (for comparison of non-parametric data). Data were presented as mean ± SE, and p values of <0.05 were considered as being statistically significant.

RESULTS
Generation of intracellular ROS by rotenone in T98G cells
To determine the optimal rotenone concentration which could induce the overaccumulation of both superoxide and peroxide radicals intracellular, T98G cells were first treated with various concentrations of rotenone (0.5 µM, 5 µM and 50 µM) for 6 h. Fig. 1 demonstrates that rotenone could induce the production of both superoxide radicals
(measured using DHE) and hydrogen peroxide (measured using DCFH-DA) in T98G cells. When compared with the DMSO-treated T98G cells, superoxide levels were considerably enhanced in T98G cells treated with rotenone at a concentration of 0.5 µM (1.37-fold, p<0.05) and gradually increased at the concentration of 5 µM (1.56-fold, p<0.05) and 50 µM (1.97-fold, p<0.01). In addition, we could demonstrate in this study that high-dose rotenone could also significantly increase the peroxide level (1.77-fold, p<0.01 at 50 µM).

**Effect of rotenone treatment on cell survival**

In this study, we investigated the effect of rotenone treatment on the survival of T98G cells. To analyze T98G cell viability following rotenone treatment, an MTS assay was performed. The result demonstrated that the viability of T98G cells treated with rotenone of either 5 or 50 µM was lower Fig. (~0.5 or 0.7-fold, p<0.05) than their counterparts treated with DMSO (Fig. 2a). These data were consistent with the superoxide level, revealing that superoxide radicals generated by rotenone were able to lessen T98G cell viability. To verify whether the decrease of T98G cell viability was triggered by the reduction of cell proliferation or induction of cell death, we performed BrdU assay for cell proliferation and TUNEL assay for cell apoptosis. The result demonstrated that T98G cells proliferation following rotenone treatment was slightly decreased (~0.9-fold; p<0.05) compared with the cells treated with DMSO (Fig. 2b).

In contrast to the result of cell proliferation, TUNEL assay in T98G cells treated with high-dose rotenone (50 µM) detected a remarkable increase of dark brown cells (~1.6-fold; p<0.05) compared with the control (non-treated cells) and DMSO-treated cells, respectively (Fig. 3). This indicates that high-dose rotenone provoked cell apoptosis which was most likely caused by overproduction of both superoxide and peroxide radicals.

**Effect of rotenone treatment on MnSOD expression**

To determine whether high-dose rotenone was efficient in stimulating the antioxidant response against the overproduction of ROS, we assessed the expression of MnSOD - a major antioxidant enzyme located in mitochondria - by gathering its level of mRNA and protein expression. As well as its specific activity levels. In mitochondria - by gathering its level of mRNA and protein expression, high-dose rotenone led to a remarkable decrease in the protein levels (~6.2-fold lower; p<0.01) and specific activity of MnSOD in T98G cells (~1.4-fold; p<0.05). This revealed that high-dose rotenone modulated the MnSOD expression in T98G cells, leading to the modulations in its mRNA, protein, and activity levels.

**DISCUSSION**

Rotenone is an herbal pesticide that blocks electrons flow from complex I to coenzyme Q of the electron transport chain in mitochondria [23]. As a lipophilic molecule, rotenone can freely penetrate through the cell membrane and inhibit the mammalian mitochondrial electron transport - could disrupt the mitochondrial structure and function. This study demonstrated that rotenone treatment in T98G cells could lead to mitochondrial damage and cell death.

![Fig. 1: Effect of various rotenone concentrations on the reactive oxygen species (ROS) production. T98G cells were treated either with various concentrations of rotenone (0.5, 5, and 50 µM, respectively) in dimethyl sulfoxide (DMSO) or with the same volume of DMSO (as vehicle) used for dissolving rotenone. ROS levels were determined using DCFH-DA and DHE assay as described under "material and methods." Fluorescence intensity of oxidized dichloro-dihydro-fluorescein diacetate and dihydroethidium in treated cells was expressed as ratio to control (cells without any treatment). All values are means, n=3. Student's t-test showed significant differences at *(p<0.05) and **(p<0.01) compared to those of DMSO-treated cells. D: DMSO, R: Rotenone](image)

![Fig. 2: Effect of rotenone treatment on cell viability (a) and proliferation (b) on various concentration. T98G cells (1×10⁶ cells) were first treated with 0.5 µM, 5 µM, and 50 µM rotenone in dimethyl sulfoxide (DMSO) or with DMSO (as vehicle) solely. Then, a MTS assay was performed for the cell viability and BrdU assay for cell proliferation, respectively, as described under the aforementioned material and methods section. Data were calculated as a percentage of the control (cells without any treatment). All values are means±standard error, n=9. Student's t-test showed significant differences at *(p<0.05) compared to the cells treated with DMSO](image)
membrane into the cytoplasm and mitochondria and easily transfer across the blood-brain barrier [23]. It has been reported that rotenone treatment could induce free radical generation, leading to oxidative damages, such as mitochondrial dysfunction, ubiquitin-dependent proteasome dysfunction and endoplasmic reticulum stress, as well as cell death [25,26]. In the present study, we confirmed that rotenone induced the accumulation of mitochondrial ROS, as reported previously [25,26]. Although superoxides were the main ROS generated as a result of mitochondrial complex I inhibition, here, we demonstrated that high-dose rotenone could stimulate the substantial production of peroxides as well. This might be due to the increased conversion of superoxides into hydrogen peroxides catalyzed by MnSOD in mitochondria.

The rotenone treatment of T98G cells performed in this study can serve as a model for oxidative stress induction during radiotherapy for glioma patients. Radiotherapy induces ROS production in tumor cells, either to facilitate tumor cell death through a process of apoptosis or to suppress the tumor growth through an inhibition of cell proliferation. Here, we found that rotenone concentration of 5 or 50 µM suppressed the viability of T98G cells, indicating high ROS levels [27]. The assessment of cell viability should be considered to be one of the primary criteria for apoptosis [28]. Through the use of a TUNEL assay, we observe an increase in DNA fragmentation which indicated cell death induced by chromatin dysfunction in T98G cells after high-dose rotenone treatment. Therefore, we suggested that the decline of cell viability following rotenone treatment is more likely due to the stimulation of cell apoptosis rather than the inhibition of cell proliferation since the BrdU assay performed in this study has indicated no significant decrease of T98G cell proliferation. Comparable results have also been reported by other previous studies, suggesting that rotenone does not affect the S-phase of cell proliferation [29,30]. Meanwhile, low-dose rotenone treatment (0.5-1 µM) for 18 h has been demonstrated to enhance apoptosis in HL-60 cells through cytochrome c release, caspase-3 activation, and DNA breakdown [26].

However, it should be considered that ROS-mediated DNA fragmentation presented by the TUNEL assay could be detected not only merely in apoptosis but also in the necrosis process [31]. In addition, using a TEM assay, we could observe the mitochondrial swelling with loss of cristae structure and loss of membrane integrity in the cells treated with high-dose rotenone, which is more likely an indicator of necrosis rather than apoptotic cell death, as described previously [32,33]. It has been suggested that an inhibitor of the mitochondrial electron transport chain such as rotenone-induced necrosis rather than apoptosis [33]. This effect was surely unexpected and should be taken into account during the management of cancer therapies in the future.
MnSOD expression in ovarian carcinoma was significantly lower compared to normal brain cells [7]. In contrast to those results, our recent report has found that MnSOD protein expression in ovarian carcinoma was significantly higher than in benign tumor and normal tissue [12].

Interestingly, the present study hinted that both the protein level and specific activity of MnSOD in the cells treated with high-dose rotenone were significantly lower than those in control cells, which contradicted findings in the previous reports [12,26]. In addition, we specifically employed the analysis of MnSOD enzyme-specific activity with a xanthine oxidase inhibition assay and inhibited Cu/ZnSOD activity by adding natrium cyanide [20]. It should be noted that the rotenone dose optimized in this study was higher than the dosages used in the aforementioned studies and presuming that the overaccumulation of intracellular ROS may exceed the antioxidant capacity of MnSOD, which reduces the availability and activity of MnSOD. More interestingly, the present study revealed that high-dose rotenone (50 µM) could modulate mRNA, protein, or specific activity levels of MnSOD in T98G cells. Although the synthesis of MnSOD mRNA has been upregulated, accumulation of intracellular ROS was elevated, as indicated by high superoxide and peroxide levels, which led to insufficient levels of active intracellular MnSOD enzyme. Thus, the imbalance between high ROS level and low activity of MnSOD found in the present study was due to high-dose rotenone-induced oxidative stress.

On the other hand, we should also consider the possibility that ubiquitin-proteasomal degradation of MnSOD protein is exaggerated by the high abundance of intrinsic ROS leading to mitochondrial dysfunction [25,26]. This assumption is supported by the TEM assay results in the present study demonstrating that the mitochondrial disruption in T98G cells is induced by high ROS levels. Alternatively, the discrepancy between high MnSOD mRNA and low protein or specific activity level might be associated with the dysregulation of MnSOD protein synthesis influenced by overproduction of ROS. The steady state of mRNA levels perceived using quantitative RT-PCR method is a cumulative result of several regulatory mechanisms, including transcription, RNA processing, and RNA stability, whereas the protein level and activity are predominantly influenced by translational and post-translational processing as well as innate variations in stability. Several comparative transcriptomics and proteomics in mouse offer evidence that there is a low correlation between transcript and encoded protein levels [43,44].

Here, we highlight the importance of the MnSOD gene expression analysis at the level of mRNA and protein, as well as enzyme activity. Unlike the previous report on ovarian cancer [12], our concomitant study has obtained that the suppression of MnSOD mRNA expression in T98G cells through siRNA transfection could reduce the protein level and enzyme activity and enhance superoxide radicals production, leading to cell death [45]. Hence, we evidently suggest that the impact of oxidative stress induced by high-dose rotenone is most likely associated with low MnSOD expression at the level of protein and enzyme activity. Increased
MnSOD mRNA levels which resulted in the increased availability of MnSOD antioxidants might be the result of a cellular adaptive response to oxidative stress. It should be considered that the impact of rotenone on cell survival is not only limited through enhancing the amount of intracellular ROS, both superoxide and peroxide radicals, but also through the modulation of MnSOD gene expression.

Based on the results of this study, we could elucidate that the discrepancy between high MnSOD mRNA and low protein or specific activity levels in human high-grade glioma cells reported in our previous study might be due to oxidative damage triggered by excessive production of ROS, as demonstrated by the increase of protein carbonyls as a marker of protein oxidative damage in the high-grade compared to low-grade glioma and normal brain cells [7]. Nevertheless, in contrast to the present study, the rate of cell proliferation was higher, and the rate of apoptosis was lower in the high-grade compared to low-grade glioma cells. It seems that the redox signaling pathway regulating cell viability was not similar to that observed in this study. It has been formerly reported that moderate ROS levels were able to induce cell proliferation through a redox signaling pathway, which contributed to tumor growth, whereas high ROS levels stimulated apoptosis [27]. Furthermore, in that study, we have demonstrated that cell survival of high-grade glioma from clinical specimens was strongly correlated with the low MnSOD mRNA level [7]. Therefore, we presume that MnSOD gene expression might be responsible for the modulation of redox signaling mechanisms contributing to tumor cell survival in high-grade glioma cells, and further affecting tumor malignancy and ROS-based cancer therapy resistance.

CONCLUSION

Based on our findings, we conclude that overproduction of ROS in rotenone-treated human GBM T98G cells could suppress the MnSOD protein level and activity even though its mRNA synthesis has increased. This diminished cell survival rates through enhancement of cell death rather than inhibition of cell proliferation. Suppression of MnSOD protein level and activity might be beneficial for radiotherapy. However, the upregulation of MnSOD mRNA synthesis during ROS-based cancer therapy should be considered, since it would maintain the availability of MnSOD activity and may also reenhance cell viability and proliferation. Therefore, the suppression of MnSOD synthesis in glioma cells before radiotherapy could be proposed as a targeted therapy to treat radiation resistance of GBM.

ACKNOWLEDGMENT

This research was funded by Riset Unggulan Universitas Indonesia (RUUI) 2010. The authors would like to express our gratitude to Direktorat Riset dan Pengabdian Masyarakat Universitas Indonesia for supporting the research and also to Higher Education Network Ring Initiative for writing assistance.

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