Analysis of Apoptosis and Cell Proliferation in Glioma Related to the Tumor Grade

Novi Silvia Hardiany1,*, Wawan Mulyawan2, Nurjati Chairani Siregar3, and Septelia Inawati Wanandi1

1Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Indonesia
2Department of Neurosurgery, Esnawan Antariksa Hospital TNI AU, Indonesia
3Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Indonesia

Introduction: Glioma is a primary brain tumor which arises from glial cells. Up to now, the cure potency of conventional glioma treatments is still low, especially in high-grade glioma. Treatment failure could be caused by cell proliferation and apoptosis inhibition. Regarding that, this research was aimed to analyze the cells proliferation and apoptosis in glioma based on the tumor grade. Methods: This was a cross-sectional study handling 21 glioma tissues which has been categorized into 5 high-grade and 16 low-grade glioma. DNA fragmentation (TUNEL) and cytochrome c analysis were performed to detect apoptosis. Glioma cell proliferation was determined using Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry assay. Results: Apoptosis in the high-grade glioma was significantly lower ($p < 0.05$) than that in the low-grade. Moreover, cell proliferation in the high-grade was higher than low-grade glioma, however it was not significant. Conclusion: Apoptosis inhibition was more prominent than the increase of cell proliferation in high-grade glioma. Therefore, novel therapeutic regimen should be developed in order to target cell apoptosis in high-grade glioma.

Keywords: Glioma, Brain Tumor, Apoptosis, Cell Proliferation, Tumor Grade.

1. INTRODUCTION

Glioma is primary brain tumor which is arises from glial cell.1 According to WHO classification, glioma are divided into low grade and high grade glioma based on the histopathology examination.2 Until now, the conventional treatments of glioma are open craniotomy surgery followed by chemo-radiotherapy.3 However, those treatments are still far from successful, especially for high grade glioma. The life expectation of high grade glioma patient is usually less than 1 year.4 Treatment failure can be caused by the high of glioma cell proliferation and inhibition of apoptosis. Uncontrolled signal growth, unlimited replication, gene mutation and abnormalities in the production and function of cycle cell regulatory proteins contribute to the enhancement of glioma cell proliferation. Inhibition of apoptosis can be caused by the action of apoptotic protein inhibitor, mutation of tumor suppressor protein such as p53 and modification of death receptor pathway.5

Previous study showed that glioma patient with the same degree of malignancy exhibited different response to radiotherapy. Reduction of tumor size in low grade glioma after radiotherapy was varies approximately 10–70% and 30–70% in high grade glioma.6 The difference of radiotherapy response perhaps is affected by the difference of cell proliferation and apoptosis at every degree of tumor. Sarkar et al. demonstrated that apoptosis index was higher in glioblastoma/high grade glioma compared to low grade glioma.7 However, other study found inverse result which exhibited the lower apoptosis index in high grade glioma compared to low grade.8 Therefore, apoptosis and cell proliferation in glioma cells is remain unclear. Regarding that, our research was addressed to analyze cell proliferation and apoptosis in glioma cells related to the tumor grade.

2. METHODS

This research was a cross sectional study conducted at Biochemistry and Molecular Biology Laboratory, Faculty of Medicine Universitas Indonesia. The samples were 21 glioma tissues derived from 21 patients at Cipto Mangunkusumo hospital, Esnawan Antariksa TNI AU hospital and Gatot Soebroto TNI AD hospital, Jakarta Indonesia. The inclusion criteria was determined for glioma patient with approving informed consent, who was diagnosed glioma by radiology examination and underwent open surgery craniotomy. If the histopathology examination did not show glioma, that patient was excluded. Glioma tissue samples were obtained by open surgery craniotomy, samples were stored at $-70 \, ^\circ \mathrm{C}$ or homogenized directly. According to histopathology examination, 21 glioma tissues samples consist of 5 high grade glioma and 16 low grade glioma samples.

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*Author to whom correspondence should be addressed.
Glioma grading of each sample was obtained from patient medical record. Ethical approval was issued by Research and Development Committee, Faculty of Medicine Universitas Indonesia (No. 409/PT.02.FK/ETIK/2008).

2.1. Apoptosis Analysis
1. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Method

Paraffin embedded glioma tissue was cut around 5 µm and attached into the slide, then deparaffinated and incubated with Proteinase-K. After that, the slide was washed with Phosphate Buffer Saline (PBS) and blocking with Hydrogen Peroxide (H₂O₂) in metanol. Washing process by PBS was performed, then incubated in Tritone solution on ice, washed two times, continued by drying process. TUNEL solution was dropped into the slide, afterward washed three times by PBS and dried. Converter-POD solution was dropped into the slide. That slide was covered by aluminium foil and incubated for 30 minutes. After incubation process, slide was washed three times by PBS and added 3,3′-Diaminobenzidine (DAB) substrate. Counter staining was performed by Hemotoxylin. Apoptosis index was calculated in percentage by counting brown cells in 200 cells under microscope examination.

2. Cytochrome C ELISA (eBioscience®) Analysis

Cytochrome C was analyzed by Enzyme-linked Immunosorbent Assay (ELISA) method. Protein in glioma tissues was extracted using cell extraction buffer. Reaction protocol was established following the manufacture instruction kit. Serial dilution of Human Cytochrom-C standard (20 ng/ml) was performed to make standard curve. One hundred micro liters of calibrator diluent (RD5P) was loaded into each well which already coated by anti-human Cytochrom-C antibody. Afterward, 100 µL of sample and Cytochrome C standard were put into each well and incubated for 2 hours in room temperature. After that, washing process using Wash Buffer was performed for 4 times. Then, 200 µl of Cytochrome-C conjugate was loaded into each well and incubated for 2 hours in room temperature. Addition the 200 µl of substrate solution into each well was performed after washing process for 4 times. Incubation of substrate solution was demonstrated for 30 minute in dark room temperature. Fifty micro liters of stop solution was added into each well and continued by reading the absorbance at 450 nm using ELISA reader.

2.2. Glioma Cell Proliferation Analysis
(PCNA Staining Kit)

Proliferating Cell Nuclear Antigen (PCNA) is cell proliferation marker that could be used for cell proliferation analysis. Cell proliferation analysis using PCNA staining kit (Invitrogen®) was performed in glioma paraffin block with Immunohistochemistry method. Reaction protocol was started with deparaffinization using xylene and continued by rehydration using graded ethanol. Tissue pre treatment was performed before staining process by soaking the slide in 95 °C Target Retrieval Solution (TRS). Staining process was demonstrated by adding blocking solution for 10 minute, PCNA primary antibody for 60 minutes at 37 °C, streptavidin peroxidase for 10 minutes and DAB chromogen for 2 minutes at room temperature. Slides were counter-stained with hematoxylin. Cell proliferation index was calculated in percentage by counting brown cells in 200 cells.

2.3. Statistical Analysis

Data was presented as mean ± Standard Error (SE) compared between low grade and high grade glioma. Statistical significance in different between groups was analyzed by Student’s t test. Statistic software used in this research was PASW 18. Significantly of statistic was set at p < 0.05.

3. RESULT

3.1. Apoptosis Analysis

Apoptotic analysis was performed using TUNEL method and Cytochrome C ELISA method. TUNEL method exhibit DNA fragmentation in the cells reflecting death cells occur via apoptosis pathway. We calculated apoptotic index as percentage by counting the dark brown cells in 200 cells. Apoptotic index by TUNEL method in high grade glioma was significantly lower (p < 0.05) compared to low grade glioma as shown in Figure 1. In order to investigate apoptosis via intrinsic pathway, Cytochrome C was analyzed. The principal of this kit is to detect translocation of Cytochrome C from mitochondria into cytosol. The result showed that Cytochrome C in high grade was lower than in low grade glioma but it was not significant (Fig. 2).

3.2. Cell Proliferation Analysis

We determined glioma cells proliferation using PCNA method. Cells proliferation index was calculated by counting the brown cells in 200 cells. We found that the glioma cells proliferation index in high grade glioma was higher compared to low grade glioma however not statistically significant (Fig. 3).

4. DISCUSSION

TUNEL reflected DNA fragmentation which is one of apoptosis characteristic in the cells. Apoptotic index that reflected cancer cell death in high grade glioma was significantly lower compared to low grade. Riberio et al. also found the same result that apoptosis index in high grade glioma was lower than in low grade one and no significant relationship with survival. Apoptosis via mitochondria pathway (intrinsic pathway) occurs by releasing of cytochrome c into cytosol to form apoptosome oligomeric apoptotic protease-activating factor-1 (APAF-1). Apoptosome will recruit and activate caspase-9, leading to activation of caspase-3 and 7. Therefore, cell death occurs through proteolysis. These pathways are usually inactive in cancer cells. In this research, we found that Cytochrome C level in high grade glioma tend to lower than low grade glioma however it was not significant. Johnson et al, proved that Cytochrome C cytoplasmic was enough to induced apoptosis in glioblastoma cell line and medulloblastoma. Low level of apoptosis in this high grade glioma meant that apoptosis was inhibited, and probably leading to the cells resist to the chemo-radiation treatment. Chemotherapy initiates apoptosis through releasing of Cytochrome C from mitochondria.

Inhibition of apoptosis in high grade glioma was accompanied with the high of cell proliferation. Cell proliferation in high grade glioma was higher compared to low grade glioma, indicating that this PCNA index could be used as a malignancy marker. PCNA is known as a cyclin, non-histone protein which is found in nuclei and have a role in cell proliferation initiation through DNA polymerase. PCNA level could increase at S, G and M

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However, certain low grade glioma sample showed the high PCNA index (data was not shown), suggesting it might have bad prognosis toward therapy. Moreover certain high grade glioma sample showed the high apoptotic index (data was not shown), suggesting it might have good prognosis toward therapy. These finding could clarify Aman’s research on the variant responses of glioma patients towards radiotherapy, where a few of the high grade glioma patients gave better response towards therapy and a few of the low grade glioma patients gave worse response towards radiotherapy. Nevertheless, further cohort study is required to analyze the relationship among cell proliferation, apoptosis and chemo-radiotherapy response.

5. CONCLUSION

In high-grade glioma, the apoptosis inhibition and cell proliferation were higher than those in the low-grade. In addition, the inhibition of apoptosis in high-grade glioma was more prominent than the increase of cell proliferation. Therefore, novel therapeutic regimen should be developed in order to target cell apoptosis in high-grade glioma.

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References and Notes