ORIGINAL ARTICLE

Expression of Senescence Markers Increased in the Frontal Lobes of Obese Rats

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ABSTRACT

Introduction: Obesity has been demonstrated to induce oxidative stress and inflammation processes that lead to senescence. Obesity-induced cellular senescence in the brain is still widely investigated. This study aimed to investigate the expression of senescence markers in the frontal lobes of obese rats. **Methods:** Three groups of rats: control, Obese-2 (Ob-2), and Obese-4 (Ob-4) were observed. Control rats were fed with a standard diet for one month. In contrast, Ob-2 and Ob-4 rats were fed with a high-fat diet daily for two and four months, respectively. After being sacrificed, the rats' brains were dissected out then the frontal lobes were used for RNA extraction. Reverse transcriptase PCR of *p-16, p-21*, and *beta-actin* was performed to investigate the relative expression of the senescence markers. **Results:** Ob-2 and Ob-4 groups had significantly increased body weight after being fed with a high-fat diet for two and four months, respectively. The mRNA expressions of *p-16* and *p-21* in the frontal lobes of three groups showed similar patterns. The ob-4 group had the highest mRNA expressions of both *p-16* and *p-21*. In comparison to control and Ob-2 groups, the mRNA expressions of *p-16* and *p-21* were markedly increased. Furthermore, the mRNA expressions of *p-16* and *p-21* between control and Ob-2 groups were comparable. **Conclusion:** Increased senescence markers in the rats' frontal lobes were observed as the chronic effect of obesity.

Keywords: Obesity, High-fat diet, Cellular senescence, Frontal lobe, Cellular senescence marker

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INTRODUCTION

Nowadays, the increased lifespan of elderly all over the world has emerged along with the burden of chronic diseases that they are suffered from. Elderly is challenged to live a longer healthy lifespan despite the normal and abnormal aging process, including the neurodegenerative diseases. On the other hand, unhealthy life style may promote the increased prevalence of obesity. Obesity leads to shorter lifespan and affects molecular processes in cellular aging.

Cellular senescence is one of the nine key hallmarks of the aging process (1,2). Cellular senescence is a cell course that halts cell-cycle progression along with the increased expression of a pro-inflammatory senescence-associated secretory phenotype (SASP) which involves in aging and age-related diseases (3). Cellular senescence, as well as apoptosis, is a physiological process to remove any damaged cells and to maintain tissue remodeling. This process is considered as an essential mechanism for tissue growth and its homeostasis. All cell types that are able to divide (replication-competent cells), such as fibroblasts, epithelial cells, melanocytes, endothelial cells, and astrocytes, may undergo cellular senescence (4-7).

Cells that undergo cellular senescence may have an enlarged flat configuration, cease DNA replication, express more abundant of proteins involved in cell cycle cessation and tumor suppression (such as tumor suppressor p53 and cyclin-dependent kinase inhibitors p16, p21, and p15), and show positive expression for SA β-gal (senescence-associated beta-galactosidase). The alteration of pro-inflammatory factors, growth factor, and proteases called senescence-associated secretory phenotype (SASP) could also be found in the senescent cells (8).

Obesity is characterized as the excess of adipose tissue accumulation. Obesity has been related to unhealthy aging, proven by its involvement in the nine key hallmarks of aging process, even its acceleration (2). It

has been showed that pre-adipocyte and endothelial cells from obese human and rats expressed more positive SA β-gal along with the increased of its activity (9). Obesity has been demonstrated to relate with neuropsychiatric disorders, such as anxiety and depression (10,11), with anxiety is shown to be behavioral characteristic in obese patients. Obesity has been proposed to have an association with neurodegenerative through damage of the central nervous system, apoptosis, and alteration of synaptic plasticity (12).

In this study, we would like to investigate whether cellular senescence, a key hallmark of aging, occurs in the brain of obese rats. We particularly observed the mRNA expressions of *p-16* and *p-21*, the cyclin-dependent kinase inhibitors as cellular senescence markers, in the frontal lobe of obese rats. Frontal lobe controls attention and executive functions in which its dysfunction may cause cognitive domain impairment in rodents, thus it may represent a neurodegenerative disease. This is a preliminary study to describe the involvement of cellular senescence in obese rats' brains for further potential research developments in the study of obesity and neurodegenerative diseases.

MATERIALS AND METHODS

Animal subjects

A total of fifteen Sprague Dawley rats (180-200 grams, young adult of 3 months-old) obtained from Experimental Animal Care Unit (UPHP) of Universitas Gadjah Mada were used for this study. The rats were housed in a cage containing up to three rats each, under the condition of room temperature 25-30°C with 50%-60% humidity, a dark-light cycle of 12:12 hours and were fed water ad libitum. The subject rats were divided into three groups, five rats each group, namely: Control, Obese-2 (Ob-2), and Obese-4 (Ob-4). The control group received standard pellet diet called AIN76A, while Ob-2 and Ob-4 groups were fed with high fat diet (HFD) 2 months and 4 months, respectively.

The rats were weighed at the start and the end of the study. After the due date of high fate diet feeding, Ob-2 and Ob-4 groups were sacrificed at day-60 and day-120, respectively. The control group was sacrificed after 2 months of standard pellet diet. The whole brain was harvested and immersed in RNA Later for mRNA assay (Ambion, 7021). The left and right frontal lobes were dissected following the neuroanatomical mapping of the rats' brains right before the RNA extraction. This study was approved by Ethical Committee of Medical Research and Health of Faculty of Medicine Universitas Gadjah Mada with ethical expediency number KE/FK/0490/EC/2018.

RNA Extraction and cDNA synthesis

Frontal lobes' tissue was extracted using Genezol RNA Solution (GENEzol™, Cat. No. GZR100) according to the manufacturer's instructions. The RNA concentration was quantified using nanodrop.

Reverse-transcription for cDNA synthesis was performed using Revertra Ace kit (Toyobo, Cat. No. TRT-101), random primer (TAKARA, Cat. No. 3801), and deoxyribonucleotide triphosphate (dNTP) (Takara, Cat. No. 4030) in a total volume of 20 µl containing 1 µg of total RNA, with PCR condition according to the manufacturer's instruction: 30°C for 10 min (annealing), 42°C for 60 min (elongation) and 99°C for 5 min (enzyme deactivation).

RT-PCR of senescence markers

Reverse Transcriptase-PCR of *p-16* and *p-21* was performed to examine the expression of senescence marker in the frontal lobes. The RT-PCR was performed using the mixture of cDNA, Taq master mix (Bioron, Germany, Cat. No. S101705) and specific primers. The following primers sets were used to amplify *p-16* and *p-21*, respectively: Forward: 5'-TGCAGATAGACTAGCCAGGGC-3', Reverse: 5'-CTCGCAGTTCGAATCTGCAC-3' and Forward: 5'-GTGATATGTACCAGCCACAGG- 3', Reverse: 5'-CAGACGTAGTTGCCCTCCAG-3'. PCR conditions were as follows: 94°C denaturation for 10 sec, annealing at 55°C for 30 sec and extension 72°C for 1 min final extension phase end with the conditions of 72°C for 10 min. The PCR products were analyzed on 2% agarose gel along with a 100bp DNA ladder (Bioron, Germany, Cat. No. 306009). The genes' expressions were quantified using densitometry analysis of the ImageJ software. The expression of β-actin (forward 5'- GCAGATGTGGATCAGCAAGC-3' and reverse 5'-GGTGTAAAACGCAGCTCAGTAA-3'), a housekeeping gene, was used to normalize the expressions of the senescence markers. The experiments were repeated three times before data analysis.

Statistical analysis

Data were presented as mean + SE until otherwise specified. The homogeneity of the data were tested using Saphiro-Wilk normality test. The difference of end-point body weight of each groups and densitometry quantification of RT-PCR results between groups were tested using one-way analysis of variance (ANOVA). Further multiple comparisons were analysed using Tukey's post hoc analysis. All statistical analyses were performed using GraphPad PRISM version 7 (GraphPad software Inc., La Jolla, CA, USA).

RESULTS

Rats' body weight after high fat diet

The rats were weighed at the start (day-0) and at the end of the study before being sacrificed (day-60 for Groups Control and Ob-2; day-120 for Group Ob-4). There was no significant difference in the body weight of the rats at the start of the study (data not shown).

However, at the end of the study, a significantly difference was found in the body weight of the rats between groups (p<0.001). The difference was found between control and Ob-2 groups as well as control and Ob-4 groups (Figure 1). The mean body weight of Ob-2 and Ob-4 groups were comparable.

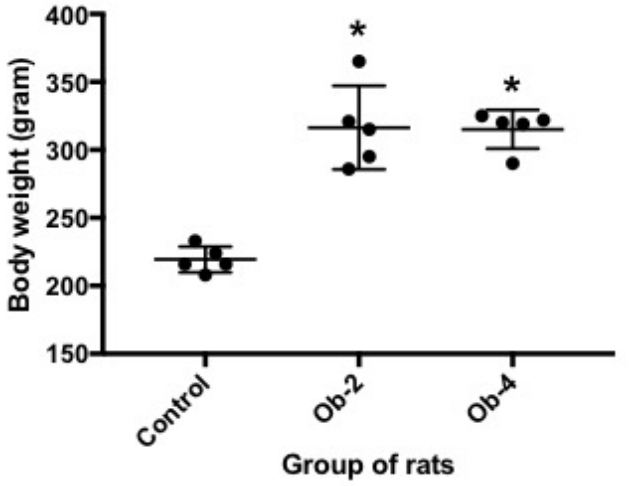

Figure 1: The Comparison of body weight between control and high-fat diet rats. Body weight of the rats after being fed with high fat diet for two and four months for Ob-2 and Ob-4 Groups, respectively. *p<0.001 vs Control

The expression of senescence markers in frontal lobes of obese rats

The size of RT-PCR products of *p-16*, *p-21*, and *β-actin* were 184, 216, and 100 base pairs, respectively. The expressions of *p-16* were markedly increased in both Ob-2 and Ob-4 groups (p<0.05) compared to Control group. Meanwhile, the expressions of *p-16* between Ob-2 and Ob-4 were comparable to each other. The expressions of *p-21* were also increased in both Ob-2 and Ob-4 groups compared to Control group (p<0.05), although only Ob-4 group showed a significant increase compared to Control group (Figure 2).

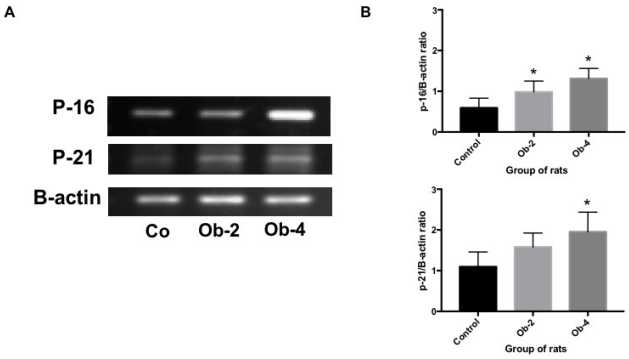

Figure 2: The expressions of *p-16* and *p-21* in the frontal lobes of obese rats. A) Representative images of gel electrophoresis from RT-PCR products of *p-16, p-21*, and *β-actin* of the three study groups. B) Relative quantification of the expressions of *p-16* and *p-21* of obese rats. The expression was normalized to the expression of the *B-actin*. *p<0.05 vs Control.

DISCUSSION

Several animal models of obesity have been introduced as monogenic and polygenic models (13,14). Frequently, rodents such as mice and rats are used as the obesity animal models with both monogenic and polygenic models, although some mammal models have also been introduced (13). Monogenic model is usually introduced by knocking out the gene involving in the satiety hormone, Leptin, its receptor, and its downstream signaling pathway in the brain. Polygenic model is introduced by diet-induced obesity. Of the two models, environmental and behavioral factors, including diet, are the primary contributors for the occurrence of obesity (14). Furthermore, outbred obese Sprague-Dawley rats have been presumed to have similar characteristics with the common form of human obesity (13). Therefore, in comparison to monogenic models, polygenic models of Sprague Dawley rats were preferentially chosen for this study.

Our study showed the mRNA expressions of cellular senescence markers both *p-16* and *p-21* in the tissue of frontal lobe of Sprague Dawley rats. This finding supported the detection of senescent cells in the brain of mammalian, where they could lead to neurodegeneration by secreting pro-inflammatory SASP or interrupting intercellular junctions for neuron-glial interaction structurally or functionally for the maintenance of neuronal ion and metabolic homeostasis (15,16). Since cellular senescence could only occur in replication-competent cells, it could be inferred that the increased mRNA expressions of both *p-16* and *p-21* were derived from glial cells, although a report stated p-2- dependent senescence feature could also be found in the neurons of aging mouse brain (17).

Several studies showed that by inducing external stress oxidative and cellular stress in vivo, astrocytes, may show characteristics of senescent cells, such as enlarged shape, elevated activity of SA β-gal, increased *p-21* and *p-16* expressions, and reduced capability to support the co-cultured neurons' viability (4,18,19). In aging brain, microglia morphologically become hypertrophic and dystrophic, lose its branches, present the formation cytoplasmic spheroids, beading, and fragmentation (20,21). As a result of external induction, microglial cells showed senescence activity by growth cessation, SA β-gal activity enhancement, and senescence-associated heterochromatic foci presentation (22). In normal and abnormal brain aging, microglial-mediated increases in pro-inflammatory SASP, including IL-1B and IL-6 (23). The senescent oligodendrocytes could cause neuronal axons demyelination, and as the aging process continues, the process of remyelination becomes ineffective (24,25). Senescent endothelial cells in the brain vessels could cause age-related disturbance in blood-brain barrier, causing influx of pro-inflammatory factors from peripheral blood flow that may contribute

in neuronal cell death (26). All these features may result in neuronal dysfunction as the prominent cause of neurodegenerative disease.

In this study, we found that mRNA expression of cellular senescence markers *p-16* and *p-21* increased in obese rats. This could imply the occurrence of cellular senescence in the frontal lobe tissue of obese rats. We inferred that the increased expressions were derived from the replication-competent glial cells, with possible minor contribution from neuronal cells. Despite its different size, the function of frontal cortex in human and rodents is quite similar as it affects attention and executive function. In rodents, the attentional and executive dysfunctions have been described as impairments on delayed response tasks in mazes or operant chambers (27). Therefore, impaired function of frontal lobe in rodent could cause the behavioral disorder, related to neurodegenerative or neuropsychiatric disorders.

Several studies reported the association of obesity and Alzheimer Disease and Parkinson Disease, some examples of neurodegenerative diseases (28-30). In some other studies, it has been found that obesity has association with neuropsychiatric disorder, particularly anxiety, with the evidence of augmentation of senescent glial cells in the brain of obese mice (31). However, whether cellular senescence in the frontal lobe caused by the chronic effect of obesity in rodent might lead to neurodegenerative or neuropsychiatric disorders has never been reported before. Despite the chronic treatment of the high-fat diet feeding to induce obesity, the use of young adult rats in this study may prevent the confounding factor of aging to contribute in senescence process. In general, comparing the age of rats to human phenotypically, rats at the age of 6 to 18 months can be defined as reproductive age ranging from late teenager to adult in human age (32). Our findings might give an additional value to the knowledge that cellular senescence might mediate the occurrence of neurodegenerative or neuropsychiatric disorders in chronic obese rats.

In obesity, it has been established that oxidative stress through the overproduction of oxidative free radicals and reactive oxygen species is an important underlying factor for the further development of many pathologies such as diabetes, cardiovascular diseases, carcinogenesis by involving DNA damage and cell cycle regulation (33). Upon the effect of oxidative stress, DNA damage, and many other stressors coming from internal or external of the cells, cellular signaling cascades involving the ultimate activation of p-53 and *p-16* pathways occurred (34). Activated p-53 induces *p-21* to target cyclin E-CDK2, causing temporal cell cycle arrest. In other pathway, activated *p-16* directly targets cyclin D-CDK4 and cyclin D-CDK6 complexes to inhibit cell cycle progression. Temporally arrested cells could undergo senescent growth arrest if the stressors received by the cell are severe. On the other hand, cells that are able to overcome mild to moderate stressors may resume cell cycle progression by activating p-53/p-21 pathway. Therefore, based on the type and level of the stressors received by the cells, p-53/p-21 pathway can either support or counteract *p-16* pathway in cellular senescence. In this study, we demonstrated the support of p-53/p-21 pathway to *p-16* pathway in promoting the cellular senescence of frontal lobe in obese rats, in which the more chronic obesity stressors may result in the more abundant expressions of CDK inhibitors in both pathways.

We realized this preliminary study has some limitations. We provided data regarding the increased cellular senescence markers in the frontal lobe of obese rats. This could suggest that the aging process in the frontal lobe of obese rats occur in accelerated fashion compared to control. Further study is necessary to establish which cell types in the frontal lobe tissue that undergoes cellular senescence in obese rats, determine what neurologic disorders caused by the obesity- induced cellular senescence in the frontal lobe by performing behavioral tests in mazes or chambers, compare the expression of cellular senescence markers in the different parts of the brain to map the distribution of obesity-induced cellular senescence in the brain and finally investigate the underlying mechanisms of the cellular senescence such as through pro-inflammatory factors or oxidative stress.

CONCLUSION

We found that cellular senescence markers were increased in the frontal lobe of obese rats as a result of chronic obesity that might mediate the obesity-induced neurodegenerative disorder.

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