An Effect of Testosterone on the Proliferation of Artificially Induced Senescent PC12 Pheochromocytoma Cells

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Backgrounds: Although the role of testosterone in the neuroprotection is still poorly known, accumulating evidence suggests that testosterone may be an important role in neurodegenerative disease including dementia. So we would like to investigate the effect of testosterone on the proliferation of senescent PC12 cells, using as a model of neural cell aging.

Methods: Rat pheochromocytoma PC12 cells from the ATCC were induced senescence artificially by AZT, the telomerase inhibitor. After 4 weeks of culture with AZT, PC 12 cells treated with testosterone overnight. The proliferating capacity of PC12 cells was determined by a difference in subcellular distribution of Ki67 expression, using as intranuclear marker for cell proliferation, and then we counted the numbers of proliferating cells which showed Ki67 IRs within nuclei to be compared with the total cell numbers.

Results: We observed Ki67 IRs in nucleus of the senescent PC12 cells with testosterone treated group and control group. In the quantitative assessment of nuclear Ki67 IR proliferating cells against total cells, the senescent cells treated with testosterone showed marked enhancement of proliferation.

Conclusion: Testosterone seems to be a protective effect in the cellular senescence in PC 12 cells by maintaining the proliferating capabilities.

Key Words: testosterone, PC12 cell, senescence, AZT

INTRODUCTION

Testosterone, the gonadal steroid hormone has long been known to function as a sex hormone, it may also play an important roles in the development and physiological function of the brain. Although the role of testosterone in the neuroprotection is still poorly known, accumulating evidence suggests that testosterone may be an option of treatment for neurodegenerative disease including dementia. The free form testosterone may cross blood brain barrier and influence neuronal cell directly. Testosterone also acts indirectly via conversion to estrogen. To distinguish androgen from estrogen neuroprotection, there are some researches going on using receptor antagonist.

Aging process of brain shows similar aspect of neurodegenerative disorder that characterized progressive dysfunction and death of neurons. As a normal consequence of aging, the testosterone levels reduced gradually. Decline of testosterone level might induce atrophy and impair compensatory responses in an insulted tissue; the aging brain becomes more susceptible to neurodegeneration.

When it comes to cellular senescence, there are only few data that testosterone have a beneficial effect or reverse the senescence process. So we would like to investigate the effect of testosterone on senescence PC12 cells, using as a model of neural cell aging, it might give us an insight about the mechanism of neuroprotective effect on the cellular basis.
MATERIALS AND METHODS

Rat pheochromocytoma PC12 cells from the American Tissue Culture Collection (ATCC) were cultured and maintained as described by the ATCC. Cells were plated at approximately $2 \times 10^5$ cm$^2$ in 24-well plates (Falcon), where coverslips for immunocytochemistry (Marienfeld, Germany) were placed. PC12 cells were cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10% heat-inactivated horse serum (Gibco-BRL, USA), 5% fetal bovine serum (Gibco-BRL, USA), and 1% antibiotic-antimycotic (Gibco-BRL, USA) at 37°C in a 95% air/5% CO$_2$ incubator. Where indicated, PC12 cells were maintained in culture medium containing 1 μM AZT (SigmaAldrich) as described$^5$.

The degree of senescence of AZT-treated PC12 cells was determined in accordance with our previous report$^6$. That is, comparing with newly plated PC12 cells, the sizes of the cells treated with AZT for 4 weeks were much larger (Fig. 1A to 1D). And after 6 weeks or more treatment of AZT, very large cells emerged (Fig. 1E and 1F), which were used in our previous study$^6$. To show the effect of testosterone on the senescent cells much clearly, we used the cells which were treated for 4 weeks, at which the cells still have proliferating capacities. After then, the cells were divided to two groups, control and testosterone group, which treated with testosterone 100 nM overnight in condition with 37°C in a 95% humidified air/5% CO$_2$ incubator.

The proliferating capacity of PC12 cells was determined by a difference in subcellular distribution of Ki67 expression. Since Ki67 is known to be a nuclear protein that is expressed in proliferating cells in G1, S, G2 and M phases and may be required for maintaining cell proliferation, it has been frequently used as a neuronal marker for cell proliferation$^7$. Used Ki67 (C-20) is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of Ki-67 of human origin (Santa Cruz, USA). The cells were washed three times with 0.05 M phosphate buffered saline and treated with 4% paraformaldehyde solution for 10 min, after which they were incubated with a 1:300 dilution of primary antibodies for 90 minutes. The cells were subsequently treated with Alexa Fluor 555 anti-goat IgG (1:500, Molecular Probes, USA) for 1 hour and the immunoreactive signals were observed with a fluorescence microscope (Zeiss, Germany). We counted the numbers of proliferating cells which showed Ki67 IRs within nuclei to be compared with the total cell numbers which could be possible by counting DAPI stained nuclei number. And then take an average ratio of each control and testosterone treated group (Ki67 expressed cell numbers × 100 /total cell numbers), and draw a graph.

Fig. 1. Comparison of the cell sizes treated with AZT during different periods. (A) and (D) PC12 cells which were plated on the dish. (B) and (E) After 4 weeks of azidothymidine (AZT) treatment, the cells size grows larger than the cells shown in (A) and (D). (C) and (F) After 6 weeks of AZT treatment, very large cells (arrows in C and F) which include multiple nuclei emerge. (D), (E) and (F) are the magnified images of (A), (B) and (C), respectively. Scale bars, (A) to (C) 80 μm, (D) to (F) 20 μm.
RESULTS

In our immunohistochemical study using anti Ki67 antibody, the subcellular localization of the protein is different in the cells which were with or without testosterone. In Figure 2, we observed Ki67 immunoreactivities (IRs) mainly within cytoplasm even though much fewer cells expressed IRs within nuclei. On the other hand, in case of the senescent PC12 cells which were treated with testosterone, localization of Ki67 IRs within nuclei were much easily observed (Fig. 3). The average ratios of control and testosterone group show below.

Fig. 2. Ki-67 expression in senescent PC12 cells without testosterone treatment. (A) and (D) Most of Ki67 IRs were localized in the cytoplasm even though very few cells exhibit the IRs within their nuclei (yellow arrows). (B) and (E) Similar finding was also identified in this plate. (C) and (F) Though most of the cells in (F) seem to be under division, Ki67 IRs were not intensely localized, which could suggest some problems during the division of these cells. (D), (E) and (F) are the magnified images of (A), (B) and (C), respectively. Scale Bars, (A) to (C) 40 m, (D) to (F) 20 m.

Fig. 3. Ki-67 expression in senescent PC12 cells with testosterone treatment. (A) and (D) Most of Ki67 IRs were localized in the nuclei (yellow arrows). (B) and (E), (C) and (F) Similar finding was also identified in this plate. (D), (E) and (F) are the magnified images of (A), (B) and (C), respectively. Scale Bars, (A) to (C) 40 m, (D) to (F) 10 m.

Testosterone(−): (Ki67 IR cell #: 100/total cell#: 559) x 100 = 17.89
Testosterone(+): (Ki67 IR cell#: 211/total cell#: 801) x 100 = 26.34

In Figure 4, we summarized the ratio of proliferating cell number (Ki67 IRs within nuclei) to total cell number within high power field.

DISCUSSION

Testosterones have a various effect on numerous organs including brain. Beyond its reproductive function, testosterone has a effect for increased muscle mass, sexual function and libido, body hair and decreased risk of osteoporosis\(^1\). Furthermore in elderly patient with frailty, their main pathophysiology thought to be sarcopenia, testosterone considered to be a important hormone. But
Testosterone Effects on PC12 Cells

![Graph showing proliferating cell number in Testosterone(-) and Testosterone(+) conditions.](image)

**Fig. 4.** Quantitative assessment of nuclear Ki67 IRs on the proliferating cells against total cells. The senescent cells treated with testosterone showed marked enhancement of proliferation.

until now, the testosterone action with neuroprotection is still poorly understood. Also the testosterone levels decline with normal aging and it seems to be potential roles with normal brain aging phenomenon. In this study we’d like to investigate how testosterone has a beneficial influence with maintenance of proliferation potential of neural cell in the cellular senescence process using PC12 cells.

While artificial senescence is not completely identical to the physiological aging process, normal diploid cells in culture may achieve a non-dividing state termed senescence after they divide a certain amount of times. This senescence seems to be due to the loss of the telomere, which is composed of specific repeats at the chromosomal ends. Therefore, a reverse transcriptase, called telomerase, which can synthesize telomeres at chromosomal ends, is crucial for bypassing cellular senescence and tumor progression⁹. Long term exposure to 3'-azido-3'-deoxythymidine (AZT), a telomerase inhibitor, has been found to induce an irreversible shortening of the telomere in certain types of cells¹⁰, and to induce cells to express morphological and biochemical signs of senescence¹¹. At passage 34, F3II cells acquire the round and enlarged morphology characteristic of senescent cells. When immunohistochemically stained for senescence-associated β-Gal (SA-β-Gal)¹², AZT-treated cells showed positive staining patterns whereas control cells showed no staining¹³.

Although this study used cells artificially induced to senescence, our previous study suggest that the distribution of ER-α IR within cells may be altered in accordance with their physiological status, including highly proliferative, well differentiated and senescent stages. At that time, we observed that the PC12 cells treated with AZT during 2 months displayed the change in subcellular localization of ER IRs as well as the morphological and biochemical characteristics of senescence. So in this study we can use AZT induced senescent PC12 cells as a model of aging cell.

In this study we also used Ki67 immunostaining a marker for cell proliferation. Ki67 is an intranuclear antigen that is exclusively expressed in all phases of proliferating cell cycle (G1-, S-, G2-, and M phase), but is absent from resting (G0- phase) cells⁷. In this study, Ki67 expression in senescent PC12 cells without testosterone treatment was localized in the cytoplasm even though very few cells exhibit the IRs within their nuclei. Though most of the cells seem to be under division, Ki67 IRs were not intensely localized, which could suggest some problems during the division of these cells on the result of senescence. But in senescent PC12 cells with testosterone treatment, most of Ki67 IRs were localized in the nuclei, and also in the quantitative assay, senescent PC12 cells treated with testosterone showed marked enhancement of proliferation. It means testosterone may have some protective effect on the cellular senescence process.

In summary, we have shown here that intranuclear Ki67 IRs increases during AZT-induced senescence in the testosterone treated PC 12 cells. This may represent testosterone seems to be a protective effect in the cellular senescence in PC 12 cells by maintaining the proliferating capabilities.

REFERENCES


