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RESEARCH ARTICLE

Autophagy May Mediate Cellular Senescence by Nicotine Stimulation in Gingival Fibroblasts

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Background: When cells are damaged by nicotine, cellular senescence due to oxidative stress accelerates. In addition, stress-induced inflammatory response and cellular senescence cause the accumulation of damaged organelles in cells, and autophagy appears to remove them. Conversely, when autophagy is reduced, harmful cell components accumulate, and aging is accelerated. This study aimed to determine the association between nicotine-induced cellular senescence and autophagy expression patterns in human gingival fibroblasts.

Methods: Cells were treated with various concentrations of nicotine (0, 0.1, 0.5, 1, 2, and 5 mM) and 10 nM rapamycin was added to 1 mM nicotine to investigate the relationship between autophagy and cellular senescence. Cell viability was confirmed using WST-8 and the degree of cellular senescence was measured by SA-β-gal staining. The expression of the inflammatory proteins (COX-2 and iNOS) and autophagy markers (LC3-II, p62, and Beclin-1) was analyzed by western blotting.

Results: The cell viability tended to decrease in a concentration-dependent manner. COX-2 showed no concentration-dependent expression and iNOS increased in the 0.5 mM nicotine treated group. The degree of cellular senescence was the highest in the 1 mM nicotine treatment group. In the group treated with rapamycin and nicotine, the conversion ratio of LC3-II to LC3-I was the highest, that of p62 was the lowest, and the level of Beclin-1 proteins was significantly increased. Furthermore, the degree of cellular senescence was reduced in the group in which rapamycin was added to nicotine compared to that in the group treated with nicotine alone.

Conclusion: This study provides evidence that autophagy activated in an aging environment reduces cellular senescence to a certain some extent.

Key Words: Autophagy, Cellular senescence, Gingival fibroblasts, Nicotine, Periodontal disease

Introduction

1. Background

Periodontal disease is a chronic inflammatory disease caused by bacteria and plaque build-up around teeth, that induces an inflammatory immune response that can damage the periodontal ligament and alveolar bone[1]. Periodontal tissue is mainly composed of gingival fibroblasts, which produce fibers and a matrix of connective tissue, and also produce DNA and proteins[2]. Therefore, gingival fibroblasts have been used extensively in periodontal-related studies. Smoking is well known as a major risk factor for periodontal disease, and there are studies that nicotine, a major toxic component of tobacco, accumulates in periodontal tissue[3]. Nicotine inhibits the viability, adhesion, and proliferation of gingival fibroblasts and increases intracellular oxidative stress[4].

Cellular damage causes oxidative reactions in cells, lipids, and proteins, leading to cellular senescence[5]. In addition, the inflammatory response is continuously activated by enzymes such as cyclooxygenase2 (COX-2), inducible nitric oxide synthase (iNOS), NADPH oxidase, and

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xanthine oxidase promotes cellular senescence[6,7]. Aged cells can be identified through senescence-associated β-galactosidase (SA-β-gal) activity, a biological marker of cellular senescence[8]. Inflammation occurs due to stress, and when damaged proteins and organelles accumulate due to cellular senescence, autophagy removes them[9].

Autophagy is an important pathological process associated with aging, such as metabolic disorders, cardiovascular disease, and neurodegeneration, and is known to be related to cellular senescence[10]. Autophagy has been suggested to play a pivotal role in the regulation of cellular senescence because the function of autophagy decreases, as harmful cellular components accumulate and senescence accelerates[11]. According to previous studies, the regulation of autophagy could be a new therapeutic approach in periodontal disease, and further details of the relationship between autophagy and aging should be continuously elucidated[12]. Recently, many studies have been conducted on autophagy and aging; however domestic studies on gingival fibroblasts are very unusual.

2. Objectives

This study aimed to investigate the expression pattern and association between cellular senescence and autophagy in gingival fibroblasts treated with nicotine.

Materials and Methods

1. Ethics statement

This study did not require IRB review because it was an experimental study using commercially available cells.

2. Study design

1) Cell culture

The human gingival fivroblast-1 (HGF-1) cell line was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, Glendale, AZ, USA) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 100 unit/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. The cell medium was replaced every 3 days.

2) Cell viability assay

The viability of HGF-1 cell was measured using the water-soluble tetrazolium-8 (WST-8; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) assay (MediFab, Seoul, Korea). HGF-1 cells were seeded in 96-well plates at an equal density of 1×10^4 cells/well and cultured for 24 hours and 48 hours at various concentrations of 0.1, 0.5, 1, 2, amd 5 mM nicotine at 37°C in 5% CO_2 humidified incubators. Each well add 5 μl WST-8 solution for 2 hours, and the OD value of the 450 nm spectrum was measured using a microplate reader (Multiscan GO; Thermo Scientific, Waltham, MA, USA).

3) Senescence associated beta-galactosidase staining

The activity level of β-galactosidase, a marker of senescent cells, was measured using an SA-β-gal staining kit (Cell Signaling Technology, Danvers, MA, USA). HGF-1 cells were seeded in 24-well plates at density of 4×10^4 cells/well, exposed to nicotine for 48 hours and 72 hours, washed once with phosphate buffered saline (PBS), and fixed with 1×fixative solution at room temperature for 1 hour. The cells were then rinsed with PBS and incubated with an adequate volume of SA-β-gal solution at 37°C overnight. Finally, stained cells were observed under a digital microscope at 100× magnification. To quantify SA-β-gal activity, the number of cells was counted by determining the cell area at random, and the percentagae of positively stained blue cells versus total cells was counted.

4) Western blot analysis

Cellular proteins were extracted using pro-prep (iNtRON Biotechnology, Seongnam, Korea) and quantified using the Bradford assay, according to the manufacturer's protocol. Subsequently, protein samples were added to 2×sample buffer and boiled for 10 minutes. Following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred onto PVDF membranes (Millipore Co., Milford, MA, USA). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour and immunoblotted with primary antibodies targeting iNOS, COX-2, LC3-Ⅱ, p62, Becilin-1, and β-actin (Cell

Signaling Technology). Secondary anti-mouse and anti-rabbit antibodies were blotted, and the signals were detected using an ECL detection reagent (Bio-Rad, Hercules, CA, USA). Protein bands were analyzed using Microchemi (D.N.R, Jerusalem, Israel).

5. Statistical analysis

All data are presented as the mean±standard deviation from at least three replicates. The means of the treatment groups were compared with those of the control group using Student's t-test. Analyses were performed using in SPSS Statistics 25 (IBM Corp., Armonk, NY, USA). Statistically significance was set at $p < 0.05$.

Results

1. Effects of nicotine on cell viability and pro-inflammatory cytokines expression

To measure the effect of nicotine on HGF-1 cells, we first determined the cell viability of nicotine at various concentrations (0.1, 0.5, 1, 2, and 5 mM) (Fig. 1A). In all experimental groups, as the nicotine concentration increased, a dose-dependent tendency was observed in which the number of cells decreased compared with that in the control group (without nicotine) until 48 hours ($p < 0.05$).

The protein expression levels of COX-2 and iNOS (Fig. 1B) were determined. There was no dose-dependent expression of either COX-2 or iNOS induced after 24 hours or 48 hours of incubation. COX-2 protein expression levels were decreased in the 0.1 mM and 0.5 mM treatment groups compared to the control group, and then

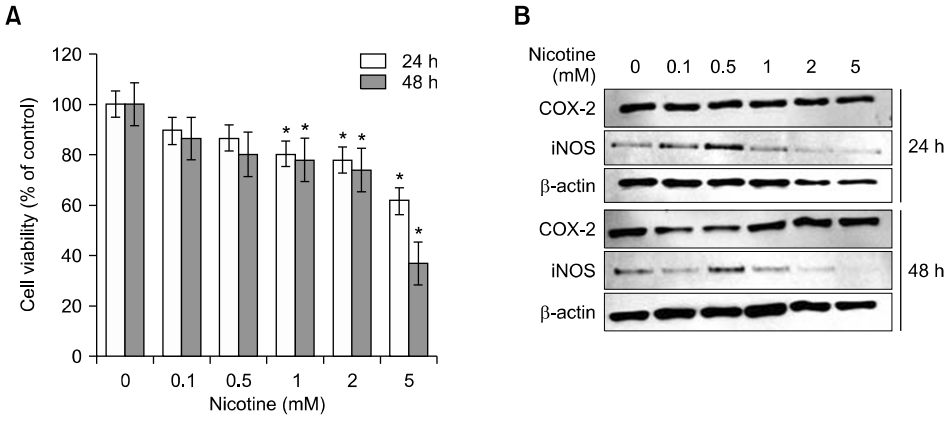

Fig. 1. Effects of nicotine on cell viability and pro-inflammatory cytokines expression in HGF-1. Cells were treated with nicotine (0, 0.1, 0.5, 1, 2, and 5 mM) for 48 hours. (A) Cell viability was estimated using WST-8. (B) The levels of protein expression were determined by western blotting. Representative data of three independent experiments are shown. *Statistically significant difference compared with control group ($p < 0.05$).

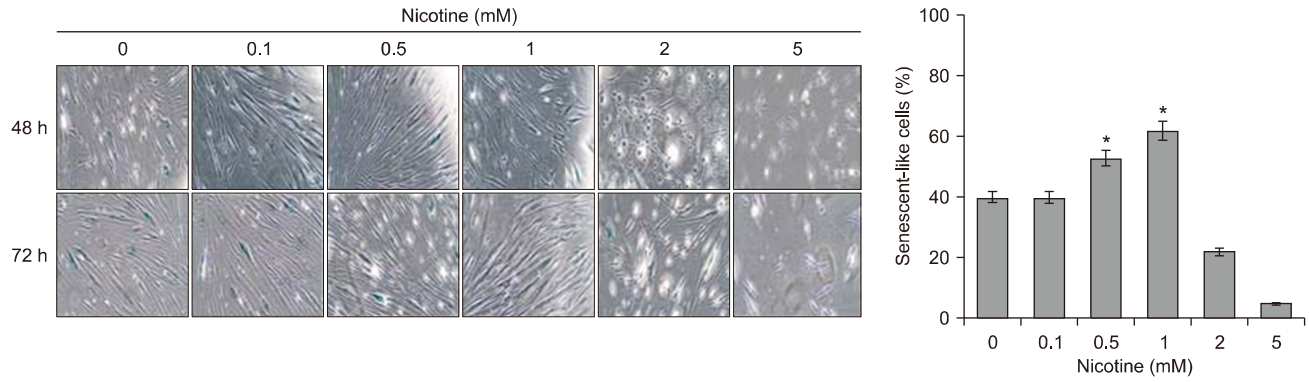

Fig. 2. Effects of nicotine treatment on cellular senescence in HGF-1. Senescence-associated β-galactosidase (SA-β-gal) staining in HGF-1 treated nicotine (0, 0.1, 0.5, 1, 2, and 5 mM) for 72 hours. SA-β-gal was used to mark the aging cells; the cytoplasm of positive cells was stained blue. Magnification ×100 and quantification of SA-β-Gal+ cells cultures. Values are mean±standard deviation of three experiments. *Statistically significant difference compared with control group ($p < 0.05$).

increased in the 1 mM treatment group. In contrast, the protein expression level of iNOS increased in the 0.5 mM treatment group and then gradually decreased thereafter.

2. Effect of nicotine treatment on senescence-associated β-galactosidase production

Next, the state of cellular senescence in HGF-1 treated nicotine was measured using SA-β-gal staining. As shown in Fig. 2, the results demonstrated that nicotine induced significant senescence of HGF-1 at concentration of 0.5 mM and peaking at 1 mM which shows 62% of SA-β-Gal+ cells ($p < 0.05$).

3. Effects of rapamycin on nicotine-induced autophagy markers and cellular senescence

The conversion ratio of LC3-II to LC3-I was highest in the experimental group treated with nicotine and rapamycin. A comparison of LC3-II protein levels revealed a decrease in the control (untreated) group and the nicotine-only group. p62 protein expression was sequentially decreased in the rapamycin-only group compared to the control group, and then increased again. Beclin-1 protein expression significantly increased in the rapamycin-treated group (Fig. 3A).

The effect of rapamycin on cellular senescence was observed by SA-β-gal staining. As a result, 65% of senescent cells were observed in the nicotine-only group, and SA-β-gal activity was higher than that in the control group ($p < 0.05$). In the experimental group in which rapamycin was added to nicotine, the number of senescent cells was significantly reduced to 47% (Fig. 3B).

Discussion

Autophagy is an intracellular degradation process that is essential for maintaining cellular homeostasis[13]. Although recent studies have suggestied that autophagy is involved in several chronic diseases and senescence, the correlation remains subject to debate. This study was designed to confirm that nicotine, one of the causes of periodontal disease, increases cell aging of human gingival fibroblasts at certain concentrations, and rapamycin, an autophagy inducer, was added to examine the expression and association of autophagy.

First, when HGFs were treated with nicotine at higher concentrations, cell viability decreased in a concentration-dependent manner (Fig. 1A). The results of the present study are consistent with the those previous studies showing that exposure of human gingival fibroblasts to nicotine reduced cell viability and increased reactive oxygen species (ROS) production in a time and dose dependent[14]. Nicotine, a pernicious ingredient in tobacco, has been reported to cause gingivitis and periodontitis[15]. As a result of confirming the degree of inflammation in

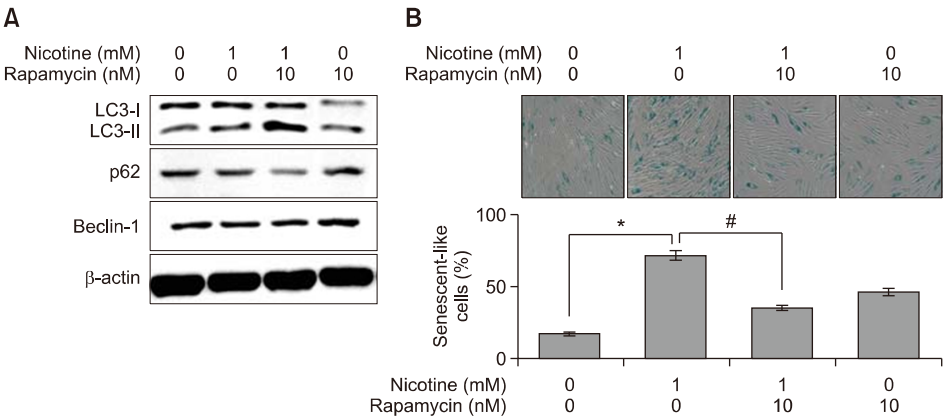

Fig. 3. Effects of nicotine and rapamycin treatment on protein expression of autophagy markers and senescence-associated β-galactosidase (SA-β-gal) staining in HGF-1. Cells were treated with nicotine (1 mM) and rapamycin (10 nM) for 48 hours. (A) Protein expression of autophagy markers (LC3-II, p62, and Beclin-1) in HGF-1 was determined by western blot analysis. Signals of each band were normalized to the respective β-actin. (B) Magnification ×100 and quantification of SA-β-Gal+ cells cultures. Values are mean±standard deviation of three experiments. *Statistically significant difference compared with control group ($p < 0.05$). #Statistically significant difference compared with nicotine treatment group ($p < 0.05$).

HGFs treated with nicotine through the expression of the inflammation-inducing proteins COX-2 and iNOS, western blotting of COX-2 showed no concentration-dependent expression. iNOS expression increased and then gradually decreased after treatment with a specific concentration (1 mM) (Fig. 1B). These findings are not in accordance with previous studies showing that nicotine induces NO production and iNOS expression in a dose-dependent manner[16,17]. However, some studies have been consistent with reported that the addition of lipopolysaccharide/interferon-gamma-NOS significantly induces the levels of iNOS and COX-2 proteins, whereas nicotine alone does not induce the levels of iNOS and COX-2 protein[18].

The degree of cellular senescence, which is known to play an important role in the development and progression of inflammatory diseases, such as periodontal disease, was evaluated using an SA-β-gal staining method based on increased β-galactosidase activity, which is commonly seen in aged cells[7,19]. In this study, compared to the control group, the highest number of senescent cells was observed (62%) in the experimental group treated with 1 mM nicotine (Fig. 2). These results were consistent with a report by Sun et al. that nicotine exposure induces aging and inflammatory secretion in mouse islet cells (Beta-TC-6) cells[20].

Rapamycin is an antifungal metabolite produced by *Streptomyces hygroscopicus*. Subseqently, it was shown to have immunosuppressive and proliferation inhibitory effects, and more research has been conducted[21]. In previous studies, rapamycin was shown to induces autophagy by significantly increasing the expression levels of Beclin-1 and LC3-II/LC3-I in human neoblastoma cells treated with rapamycin, compared to those of p62, mTOR, and p-mTOR proteins[22].

According to Tang et al., autophagy is enhanced by rapamycin significantly increasing the expression of LC3 and Beclin-1 in a rat model of acute spinal cord injury model[23]. In this study, Beclin-1 levels were not consistent with previous studies because there was no difference between the experimental group. However, in cells treated with 10 nM rapamycin and 1 mM nicotine, the conversion rate of LC3-II/LC3-I and the protein level of LC3-II itself increased compared to cells treated with only 1 mM nicotine, indicating increased autophagy in cells aged because of nicotine. In the case of p62, the protein expression was decreased in the cells in which 10 nM rapamycin was added to 1 mM nicotine compared to the control and cells treated with only 1 mM nicotine, but cells treated with only 10 nM rapamycin showed no difference from the control, which was not a reliable indicator (Fig. 3A). In addition to autophagy, p62 is involved in other intracellular and extracellular functions, regulating proteolysis by several MAPKs, and affecting antioxidant and tumorigenesis[24].

The association between cellular senescence and autophagy was confirmed through SA-β-gal staining by applying rapamycin which induces autophagy and 1mM nicotine to the experimental group. In cells treated with only 1 mM nicotine, cells treated with 10 nM rapamycin was added to 1 mM nicotine, and cells treated with only 10 nM rapamycin, blue-stained senescent cells were observed in 65%, 47%, and 59% of cells, respectively (Fig. 3B). By adding rapamycin, the number of senescent cells decreased, and cellular senescence induced by nicotine stimulation showed a tendency to decrease with autophagy inducers. This suggests that autophagy increases due to senescent cells, and that increased autophagy reduces cellular senescence, following previous studies that cellular senescence is caused by a decrease in the function of lysosomes responsible for autophagy[11].

1. Limitations

In this study, we found that autophagy activated in an aging environment reduced cellular senescence to some extent. However, there is a limitation in that the relationship between autophagy and cellular senescence cannot be accurately verified by simply changing the increase or decrease in senescent cells due to the application of an autophagy inducing agent. Further studies on the changes in other mechanisms that can measure cellular senescence, such as p16 and p21, are needed.

2. Suggestions

Nevertheless, the aim of this study was to analyze the interrelationship between inflammatory factors and cellular

senescence mechanisms to discover the evidences of the mechanism of aging, suggesting the possibility of the development of preventive and therapeutic agents for periodontal diseases.

Notes

Conflict of interest

Sang-Im Lee has been editorial board member in Journal of Dental Hygiene Science since September 2019. She was not involved in the review process of this editorial. Otherwise, there was no conflict of interest.

Ethical approval

This project does not require IRB review because it is an experimental paper using commercially available cells.

Author contributions

Conceptualization: Sang-Im Lee and Nu-Ri Jun. Data acquisition: Nu-Ri Jun. Formal analysis: Sang-Im Lee, Jong-Hwa Jang, Jae-Young Lee, and Nu-Ri Jun. Funding: Sang-Im Lee. Supervision: Sang-Im Lee. Writing-original draft: Sang-Im Lee and Nu-Ri Jun. Writing-review & editing: Sang-Im Lee.

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