Molecular Aspects of the Geroprotective Effect of Peptide KE in Human Skin Fibroblasts

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Abstract—Skin aging is an current problem of modern geriatric cosmetology. One promising method to decelerate the process of age-related skin changes is the use of cosmetics with short peptides. The goal of the work was to study the effect of peptide KE (Lys-Glu, Vilon) on the expression of aging markers of human fibroblasts in an in vitro model. The expression of collagen type 1 and sirtuin 6 was studied with immunofluorescent confocal microscopy in "young" and "old" cultures of skin fibroblasts. It has been found that the area of expression of collagen type 1 and sirtuin 6 decreases by 3.5 and 3.6 times, respectively, in skin fibroblast cultures with aging. Peptide KE increases the expression area of collagen type 1 in old skin fibroblast cultures by 83%; the expression area of sirtuin 6 in young and old cultures of skin fibroblasts increased by 1.6 and 2.6 times, respectively. Thus, peptide KE increases the functional activity of skin fibroblasts and decelerates their senescence.

INTRODUCTION

The process of skin aging is caused by epigenetic and genetic factors. Genetic structural changes in the skin are the result of the methylation of genes encoding signal molecules that regulate the functional activity of skin cells and are of an individual nature. Genetic and epigenetic aging factors cause histological changes in the skin that consist of the flattening of epidermal ridges and weakening of the surface contact between the epidermis and dermis, which leads to a deterioration of the exchange of nutrients and metabolites between skin layers.

The rate of age-related changes in the skin is not the same for men and women and depends on the hormonal background of the body. Epigenetic factors, including exposure to sunlight, environmental pollution, smoking, repetitive movements of facial muscles, and lifestyle, can be controlled through various preventive measures [3]. Short peptides are related to the agents that ensure decelerating the skin aging process. Thus, the effect of peptides KE (Lys-Glu, Vilon), KED (Lys-Glu-Asp, Vezugen), AED (Ala-Glu-Asp, Kartalaks), and AEDG (Ala-Glu-Asp-Gly, Epitalon) on the processes of proliferation (Ki67 protein), regeneration and aging (CD98hc), apoptosis (caspase-3), and remodeling of the intercellular matrix (MMP-9) in skin fibroblasts during their senescence in the culture was studied by immunofluorescence confocal microscopy. All of the studied peptides reduced MMP-9 synthesis, which is increased during the aging of rat fibroblasts, and increased the expression of Ki67 and CD98hc, the synthesis of which is decreased during cellular senescence. Peptides AED and AEDG reduced the severity of caspase-dependent apoptosism, which increased with aging of cell cultures [1].

Peptide KE is a representative of the peptide thymomimetics that was found in the composition of thymalin. In research on the immunomodulating properties of peptide KE, it was found that this peptide stimulates the cellular immunity and nonspecific resistance of the organism and exhibits a stimulating effect on macrophages and neutrophils [4, 5]. Peptide KE enhances the processes of differentiation of lymphoid cells; in addition, it showed pronounced geroprotective properties in a model of accelerated (radiation) aging of the organism and promoted an increase in animal life span [8]. At the same time, the molecu-

lar and cellular aspects of the geroprotective action of peptide KE towards human skin fibroblasts are insufficiently studied at present.

The goal of the work was to study the effect of peptide KE on the expression of aging markers of human fibroblasts in an in vitro model.

MATERIALS AND METHODS

Fibroblasts were isolated from a parotid region skin obtained as a result of a circular face lifting operation for a woman (born in 1970). After preparation, the skin was treated in sterile conditions with a solution of dyspase II at a concentration of 2.4 U/mL for 18 h at 4°C, and the epidermis was then mechanically separated from the dermis. To obtain a cell suspension, the dermis was minced with scissors to pieces of 2–3 mm in size and placed in a solution of collagenase type I in M199 medium. The resulting cell suspension was precipitated at 1000 rpm for 5 min, after which the cell pellet was resuspended in a nutrient medium. The nutrient medium consisted of M199 medium, 10% fetal bovine serum, 1% L-glutamine, 1.5% HEPES buffer, and a 1% penicillin–streptomycin solution (PanEko; a 5-mL bottle contains: penicillin 5000 U/mL, streptomycin 5000 μg/mL, and 0.9% NaCl as a solvent). After 5 days, the primary culture reached the monolayer and was reseeded in a 1 : 3 ratio. A trypsin–versene 1 : 1 solution (sterile, ready for use in 200-mL bottles, Biolot) was used to remove the cells from the substrate. An amount of 500 μL was added to each bottle (volume of 50 mL, area 25 cm², sterile, with adhesive surface and ventilated cap; CellATTACH, Jet Biofil), and the action time was 3 min at 37°C. Cellular detachment from the substrate was monitored under a microscope. A complete nutrient medium was added (5 mL per bottle) to block the enzymatic reaction. The cell suspension was then centrifuged for 5 min at 1000 rpm. The cell concentration for the zero passage was 50000 cells per mL of medium in one well of a 24-well plate (sterile, with an adhesive surface, CellATTACH, Jet Biofil). Cell passaging was performed three days later, on the fourth day, when the culture reached the monolayer. Cultivation was carried out until the third and 14th passages, on which the cells were sown on the plate.

The cells of the third and 14th passages were divided into three groups: Group 1, control cultures (without peptide); Group 2, cultures supplemented with peptide KE at a concentration of 20 ng/mL; Group 3, cultures supplemented with control pancreoprotective peptide KEDW (Pankragen, H-Lys-Glu-Asp-Trp-NH$_2$) at a concentration of 20 ng/mL [7]. A peptide concentration of 20 ng/mL was chosen, because the other short peptides at this concentration were most effective towards skin fibroblasts in previous studies [1]. In accordance with the recommendation of the International Association for Cell Culture

Research (San Francisco, 2007), the third passage was regarded as "young" cell cultures; the 14th were considered "old" cell cultures. Immunocytochemical staining of the cultures was then carried out. To carry out permeabilization, 0.1% Triton X-100 (Biolot) dissolved in phosphate buffered saline was used. Cell cultures were then incubated in 1% phosphate buffered saline (pH 7.5) for 30 min to block nonspecific binding. Incubation with primary antibodies was carried out for 60 min. Primary monoclonal antibodies to collagen type 1 (1 : 100; Abcam, United States) and sirtuin 6 (1 : 200; Abcam, United States) were used in the work.

Confocal microscopy of cells was carried out on an Olympus Fluoview CM FV300-IX70 inverted confocal microscope with a 606 UPlan apochromatic lens. A 488-nm excitation wave of an argon laser was used to specify the fluorescence of the test markers. The cell nuclei were stained with Hoechst 33258 (Sigma); as a result, they fluoresced in dark blue. Green or red fluorescence characterized the expression of the test markers (incubation with secondary antibodies conjugated with Alexa Fluor 488 (1 : 1000; Abcam) or Alexa Fluor 647 (1 : 1000, Abcam) fluorochromes for 30 min at room temperature in the dark. The finished preparations were placed under cover slips in Dako Fluorescent Mounting Medium (Dako, United States).

VideoTest-Morphology 5.2 software was used to analyze the results, as described earlier [6, 10]. In each case, five viewing fields were analyzed at a magnification of 200. The expression area was calculated as the ratio of the area occupied by immunopositive cells to the total area of cells in the viewing field and expressed as a percentage. This parameter characterizes the number of cells in which the test marker is expressed. The optical density of expression, which reflects the amount of the test marker synthesized in one cell, was evaluated in arbitrary units.

Statistical data processing included the calculation of the arithmetic mean, standard deviation, and confidence interval for each sample; data processing was carried out in the Statistica 6.0 program. The Shapiro–Wilk test was used to analyze the distribution type. Nonparametric procedures for one-way analysis of variance (the Kruskal–Wallis test) were used to assess the statistical homogeneity of multiple samples. When the analysis of variance revealed a statistically significant heterogeneity of several samples, procedures for multiple comparisons with the Mann–Whitney U test were used for the subsequent identification of heterogeneous groups (by their pairwise comparisons). The critical level of reliability of the null hypothesis (about the absence of differences) was assumed to be 0.05.

RESULTS AND DISCUSSION

The area of the expression of collagen type 1 in old cultures of skin fibroblasts was 3.5 times lower than in

Table 1. Effect of peptides on the expression area of aging markers of skin fibroblasts

Marker	Expression area, %					
	"young" cultures			"old" cultures		
	control cultures	peptide KE	peptide KEDW	control cultures	peptide KE	peptide KEDW
Collagen type 1	27.22 ± 2.78	29.75 ± 1.10	26.13 ± 2.98	7.92 ± 1.51*	14.54 ± 1.17**	8.16 ± 1.76
Sirtuin 6	8.54 ± 1.16	13.69 ± 0.73**	6.79 ± 2.00	2.37 ± 0.31*	6.12 ± 0.63**	2.03 ± 0.42

Here and in Table 2, * $p < 0.05$ as compared with the corresponding indicator in the control in young cultures; ** $p < 0.05$ as compared with the corresponding indicator in the control.

Table 2. Effect of peptides on the optical density of the expression of aging markers of skin fibroblasts

| Marker	Optical density, arbitrary units					
	"young" cultures			"old" cultures		
	control cultures	peptide KE	peptide KEDW	control cultures	peptide KE	peptide KEDW
Collagen type 1	34.7 ± 1.8	37.9 ± 0.9**	32.2 ± 0.8	31.4 ± 2.2	38.0 ± 2.1**	31.6 ± 1.8
Sirtuin 6	20.1 ± 2.3	26.5 ± 1.4**	21.3 ± 1.4	7.6 ± 0.5*	10.9 ± 0.6**	7.3 ± 0.7

young cultures (Table 1). At the same time, the optical density of expression of collagen type 1 in young and old cultures of skin fibroblasts did not differ significantly (Table 2). The obtained data indicate that the number of skin fibroblasts in which an active synthesis of collagen type 1 occurs is reduced with aging. Peptide KE increased the expression area of collagen type 1 in old fibroblast cultures by 83% but did not affect this indicator in young cultures (Table 1, Fig. 1). Peptide KE increased the optical density of expression of collagen type 1 in young and old fibroblast cultures by 9 and 21%, respectively (Table 2). Peptide KEDW tropic to pancreatic tissue did not affect the area and optical density of collagen expression in cultures of skin fibroblasts during aging.

The expression area of sirtuin 6 in the old fibroblast cultures was 3.6 times lower than in young cultures (Table 1). The same trend was observed for optical density. The optical density of the sirtuin 6 expression in old cultures of skin fibroblasts was 2.6 times lower than in young cells (Table 2). The data indicate that the number of skin fibroblasts in which sirtuin 6 is synthesized decreases with aging. Peptide KE increased the expression area of sirtuin 6 in young and old cultures of fibroblasts by 1.6 and 2.6 times, respectively (Table 1). Peptide KE increased the optical density of the sirtuin 6 expression in young and old cultures of fibroblasts by 1.3 and 1.4 times, respectively (Table 2). The KEDW peptide did not affect the area and optical density of sirtuin 6 expression in skin fibroblast cultures during aging.

CONCLUSIONS

The specific proportion of collagen in the skin decreases with chronological aging and photoaging. orphometric studies of abdominal skin in patients of different age groups (10−75 years) revealed an age-related progressive decrease in the proportion of connective tissue containing collagen fibers. These changes were more pronounced in the middle part of the dermis and reached significant values by the age of 50. In this case, the synthesis of collagen type 1 and type 3 is decreased [2]. However, the rates of reduction in the synthesis of collagen type 1 is more significant; therefore, the ratio of collagen type 3 to collagen type 1 (in young patients, by 15 and 80%, respectively) is changed, which correlated with the age of patients [12]. These data are consistent with our results, which indicate that the area of expression of collagen type 1 in old fibroblast cultures is 3.5 times lower than in young cultures. In this case, peptide KE can partially slow the age-related decrease in collagen synthesis in human fibroblasts and increase their functional activity.

Sirtuin 6 (SIRT6) protein is a critical regulator of genome stability transcription, telomere length, DNA repair, and metabolic homeostasis [11]. Sirtuin 6 modulates telomeric chromatin, deacetylates lysine 9 of histone H3, and prevents telomeric dysfunction and early cellular senescence [9]. A decrease in the synthesis of sirtuin 6, which we found in skin fibroblasts with aging, and an increase in the expression of this protein under the action of peptide KE may indicate a promising application of this peptide as a geroprotector in cosmetology.
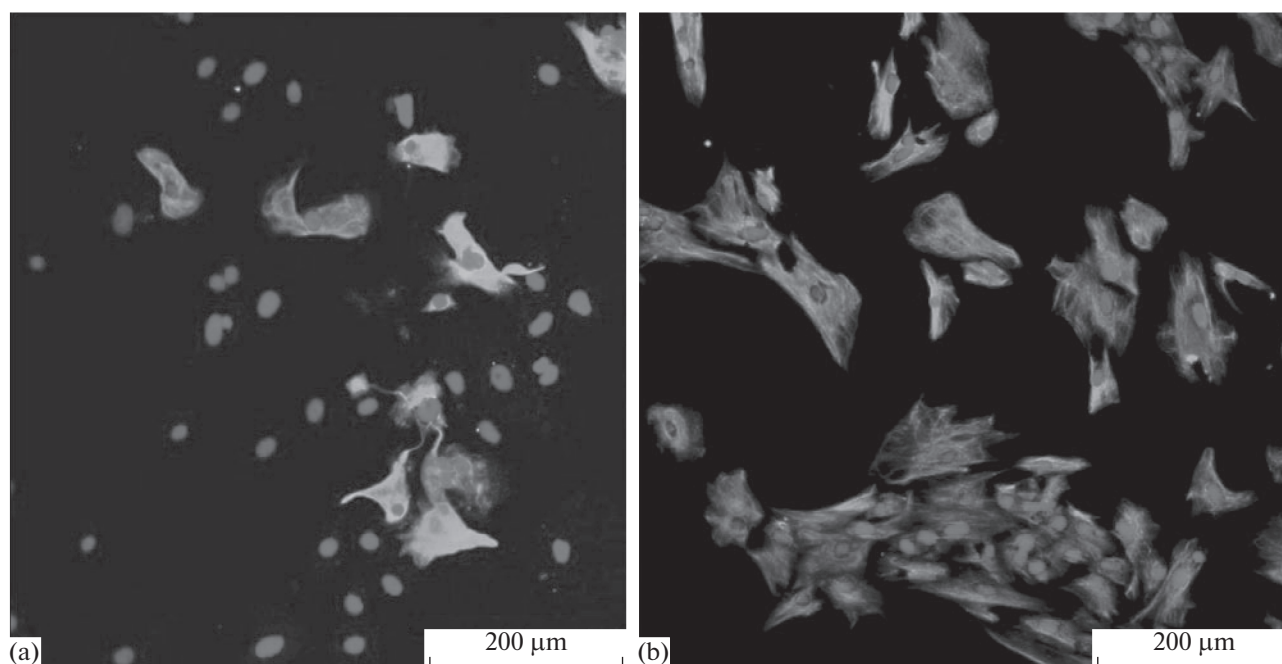
Fig. 1. Expression of collagen type 1 in skin fibroblasts culture in the 14th passage (old culture): *a*, control; *b*, peptide KE. Immunofluorescent confocal microscopy, magnification 200x; the cell nuclei were stained with Hoechst 33258, dark blue fluorescence; staining for collagen type 1, green fluorescence.

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SPELL: 1. collagenase