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Peptide Regulation of Skin Fibroblast Functions during Their Aging *In Vitro*

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The effect peptides KE, KED, AED and AEDG on proliferation (Ki-67), regeneration and aging (CD98hc), apoptosis (caspase-3), and extracellular matrix remodeling (MMP-9) in skin fibroblasts during their aging in culture were studied by immunofluorescent confocal microscopy. All studied peptides inhibited MMP-9 synthesis that increases during aging of skin fibroblasts and enhanced the expression of Ki-67 and CD98hc that are less intensively synthesized during cell aging. Peptides AED and AEDG suppressed caspase-dependent apoptosis that increases during aging of cell cultures.

Key Words: *short peptides; skin; aging; signal molecules; cell cultures*

Aging in the skin that performs barrier function is determined by damaging effects of environmental factors and deceleration of cell renewal processes in the body. Age-related changes in skin fibroblasts are primarily associated with suppression of the synthesis of collagen, hyaluronic acid, and other bioactive substances [2,4]. The balance between apoptosis and proliferation of skin cells is controlled by interleukins (IL-1, IL-2, IL-3, and IL-4), growth factors (FGF, TNF-α, *etc*.), and other signaling molecules produced by fibroblasts and vascular endothelial cells [1]. Caspase-3 and Ki-67 are most commonly used markers for evaluation of apoptosis and proliferation of skin cells. Caspase-3, the principal component of the apoptotic cascade, is involved in apoptotic chromatin condensation and DNA fragmentation in all types of cells including skin fibroblasts. Moreover, this marker is expressed in the cell at the stage preceding apoptosis. Thus, caspase-3 expression can be used for quantitative evaluation of apoptosis in skin fibroblasts [11,12]. Expression of Ki-67 protein is associated with cell proliferation. Ki-67 is detected in the nuclei of G1, G2, S and M phase proliferating skin cells and other cell types; its expression attains maximum in G2 and M phases of the cell cycle. In resting cells (G0 phase), this protein is absent. The level of Ki-67 expression is an informative indicator of the intensity of skin fibroblast proliferation [8-10,15].

Marker CD98hc is known as a molecule involved in skin renewal process. Knockout of the gene encoding CD98hc disturbs proliferation and migration of skin cells and impairs healing process. Similar changes in skin fibroblasts are observed during natural aging, which correlates with the age-related decrease in CD98hc expression. Thus, the level CD98hc expression can be used for evaluation of regeneration capacity of skin fibroblasts [7].

Matrix metalloproteinase-9 (MMP-9) implements remodeling of the extracellular matrix by skin fibroblasts. UV-induced synthesis of MMP-9 promotes destruction of type I and III fibrillary collagen in the derma. Coexpression of MMP-2, MMP-3, and MMP-9 leads to degradation of non-collagen components of the derma, including glycoproteins and basement membrane proteoglycans. Aging of skin cells is associated with a decrease in the level of tissue MMP inhibitors, which contributes to activation of extracellular matrix remodeling [13,14].

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Short peptides AEDG, KED, KE, and AED were designed and synthesized at the St. Petersburg Institute of Bioregulation and Gerontology.

AEDG peptide synthesized on the basis of amino acid analysis of the polypeptide complex of the pineal gland exhibits antioxidant properties in cell cultures and in animal experiments, regulates melatonin synthesis, demonstrates immunostimulating properties, and contributes to telomere lengthening in normal fibroblasts and overcoming the Hayflick limit [6]. All these properties of the peptide attest to its possible geroprotective effects on skin fibroblasts. Peptide KED synthesized on the basis of amino acid analysis of vascular polypeptide complex exhibited vasoprotective effect and stimulated division of endothelial cells and fibroblasts after oral administration to humans, experimental animals, and vascular endothelial cultures [4]. The biological activity of KE peptide synthesized on the basis of amino acid analysis of thymus polypeptide complex is aimed at activation of proliferation and differentiation of epithelial and immune cells of the thymus; in animal experiments, this peptide promoted wound healing [3,6]. Peptide AED designed on the basis of polypeptide complex of the cartilage regulated connective tissue metabolism in experimental animals models [2,6]. These data suggest that peptides KED, KE, AED, and AEDG can participate in the regulation of fibroblast functions.

Analysis of the efficiency of creams containing peptide AEDG showed that this peptide increases moisture content in the surface layers of the skin, visually reduces neck and face wrinkle relief in elderly women. In addition, peptides KE and AEDG stimulated cell proliferation and reduced the intensity of apoptosis in organotypic cultures of skin cells from rats of different age [4,5].

Here we compared the effect of short peptides AED, KED, KE, and AEDG on the expression of markers of cell renewal and extracellular matrix remodeling in cultures of skin fibroblast during their aging.

MATERIALS AND METHODS

Primary fibroblast cultures were derived from the skin of young (3 month-old) Wistar rats by enzymatic dissociation with collagenase. The primary cultures were isolated on Petri dishes (Sarstedt) pretreated with fibrinogen (Gibco); culturing was performed in 25 cm^2-flasks (Sarstedt) with pretreated surface. The cells were cultured in CO_2-incubator at 37°C in a medium containing 87.5% DMEM, 10% fetal calf serum, 1.5% HEPES buffer, 1% penicillin and streptomycin, and α-glutamine. The cell were subcultured (1:3-1:5) every 3 days using a mixture of 0.125% trypsin, 0.02 M EDTA, and 0.02% glucose in phosphate buffered saline (seeding density ~3×10^5 cells per flask).

Peptide solution was added to the culture after each subculturing. The cells were cultured until passages 3 and 14, and then, immunocytochemical staining was performed. Cultures of passages 3 and 14 were considered as young and aged cultures, respectively (according to recommendations of the International Association of Cell Culture Studies, San Francisco, USA, 2007). The young and aged cultures were divided into 5 groups and were grown in the presence of saline (group 1; control), AED (group 2), KED (group 3), KE (group 4), and AEDG (group 5). All peptides were added to the culture medium in a final concentration of 20 ng/ml.

For immunofluorescence staining, the cells were passaged in a 24-well plate (BioloT). Primary monoclonal antibodies to caspase-3 (1:75), Ki-67 (1:50), CD98hc (1:125), and MMP-9 (1:75) (Novocastra) were used.

The cells were permeabilized with 0.1% Triton X-100 (BioloT) dissolved in PBS. Then, the cultures were incubated in 1% PBS (pH 7.5) for 30 min to block nonspecific binding and then incubated with primary antibodies for 60 min.

Confocal microscopy was carried out using an Olympus Fluoview CM FV300-IX70 inverted confocal microscope with a 606 UPlan apochromatic lens. Fluorescence was excited with a 488-nm argon laser. Cell nuclei were poststained with Hoechst 33258 (Sigma) and they fluoresced dark blue, while light green fluorescence showed expression of the studied markers (incubation with second antibodies conjugated with fluorochrome Alexa Fluor 488 (1:1000; Abcam) for 30 min at room temperature in the dark). The images were processed using VideoTest-Morfologiya 5.2 software. In each case, at least 5 fields of view were analyzed at ×200. The area of expression was calculated as the ratio of the area occupied by immunopositive cells to the total area of cells in the field of view and expressed in percents. This parameter characterizes the number of cells expressing the studied marker.

Statistical processing of the results included calculation of the arithmetic mean, standard deviation, and confidence interval for each sample (Statistica 6.0 software). The type of distribution was evaluated using Shapiro–Wilk test. Statistical homogeneity of samples was evaluated using nonparametric univariate analysis of variance (Kruskal–Wallis test). When statistically significant non-homogeneity of samples was revealed by the analysis of variance, subsequent detection of non-homogeneous groups (by their pairwise comparisons) was performed by multiple comparison procedure using Mann–Whitney *U* test. The critical level

of significance of the null hypothesis (no difference) was set at 0.05.

RESULTS

First, we assessed the effect of short peptides on the rate of skin fibroblast division during their aging. This parameter was quantified using Ki-67 marker. In the control, expression of Ki-67 in young cultures 3.48-fold surpassed the corresponding parameter in aged cultures. Peptides AED, KED, KE, and AEDG enhanced Ki-67 expression in young cultures by 1.14, 1.89, 1.40, and 2.08 times, respectively, and in aged cultures, by 1.60, 5.61, 3.87, and 4.94 times, respectively (Fig. 1).

Then, we assessed the effect of peptides on skin fibroblast apoptosis during their aging. For quantitative evaluation of this parameter, we used caspase-3 as the marker. Expression of caspase-3 in young cultures was 3.8-fold lower than in aged cultures. Peptide AED reduced the expression of this marker in young cultures by 1.43 times, while peptides KED, KE, and AEDG did not significantly change this parameter. Peptides AED and AEDG reduced the caspase-3 expression in young cultures by 2.52 and 5.05 times, respectively, while peptides KED and KE did not significantly change this parameter (Fig. 2).

Quantitative evaluation of the intensity of extracellular matrix remodeling in skin fibroblast cultures during aging was performed using MMP-9 marker. In the control, expression of MMP-9 in young cultures was lower by 4.24 times than in aged cultures. None of the studied peptides affected MMP-9 expression in young fibroblast cultures. Under the effect of peptides AED, KED, KE, and AEDG, expression of MMP-9 in young cultures decreased by 3.43, 2.88, 4.50, and 1.29 times, respectively (Fig. 2).

Expression of CD98hc, a marker of functional activity of skin cells, in young cultures was higher by 2.92 times than in aged cultures. Peptides AED, KED, KE, and AEDG increased this parameter in young cultures by 1.66, 1.49, 1.22, and 2.94 times and in aged cultures by 2.38, 3.90, 1.62, and 6.75 times, respectively (Fig. 1).

These findings suggest that the mechanism of action of short peptides is related to activation of proliferation and deceleration of the aging process, which is seen from enhanced expression of Ki-67 and CD98hc markers under the effect of AED, KED, KE, and AEDG peptides in young and aged of skin fibroblast cultures. AEDG peptide produced the most pronounced stimulatory effect. Thus, this tetrapeptide can be studied as an active revitalizing component of cosmetic products for humans with severe signs of skin aging.

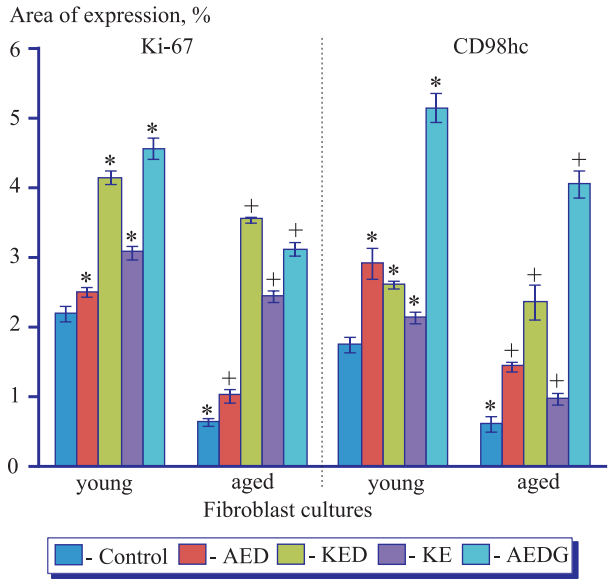

Fig. 1. Effect of peptides on proliferation and apoptosis in skin fibroblasts during their aging. Here and in Fig. 2: $p<0.05$ in comparison with the control for *young and +aged cell cultures.

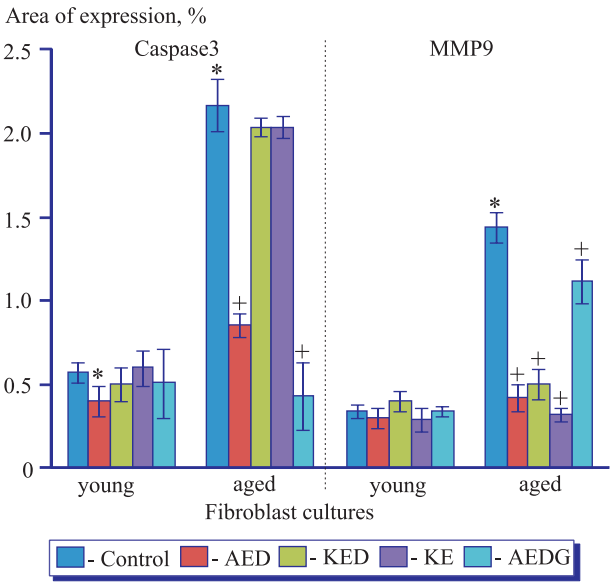

Fig. 2. Effect of peptides on cell renewal and extracellular matrix remodeling in skin fibroblast cultures during their aging.

We demonstrated enhanced expression of apoptotic marker caspase-3 in aged cultures of skin fibroblasts in comparison with young cell cultures. Peptide AED reduced the level of apoptosis in young and aged cultures, while AEDG peptide reduced this parameter in aged cultures below the level observed in young cultures (Fig. 3). Thus, peptide AED can be tested as the substance preventing skin fibroblast death for deceleration of skin aging. Peptide AEDG produced a 2-fold more pronounced geroprotective effect on aged cultures of skin fibroblasts than peptide AED, which makes it a promising agent for further study as the

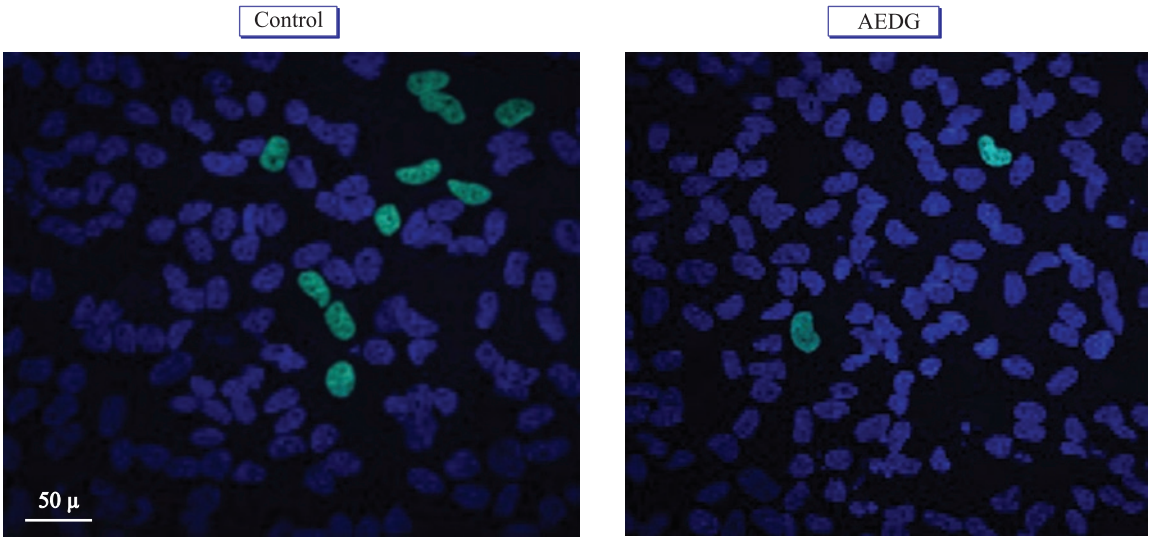

Fig. 3. Immunofluorescent confocal microscopy of skin fibroblast culture: passage 14, ×200. Cell nuclei are poststained with Hoechst 33258 (dark blue fluorescence), light green fluorescence corresponds to staining for caspase-3.

substance decelerating aging process in the skin of elderly people.

Enhanced expression of MMP-9 in old skin fibroblast cultures in comparison with young cell cultures reflected intensification of extracellular matrix remodeling and inflammatory reactions with age. Short peptides AED, KED, KE, and AEDG reduced the expression of MMP-9 in aged cultures and did not affect this parameter in young cultures. These findings suggest that at the tissue level the studied short peptides will promote skin healing, prevent irritation, increase skin turgor and hydration, and decelerate aging process.

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