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Comparison of the Effects of KE and AED Peptides on Functional Activity of Human Skin Fibroblasts during Their Replicative Aging

N. V. Fridman[1], N. S. Linkova[1,2], E. O. Kozhevnikova[1], E. O. Gutop[1], and V. Kh. Khavinson[1]

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We studied the effect of KE and AED peptides on the expression of sirtuin-1, sirtuin-6, collagen I, cytokines (IL-1, TGF-β), and transcription factor NF-κB in human skin fibroblasts during their replicative aging. Immunocytochemical analysis and confocal microscopy showed that KE peptide reduces the synthesis of factors of the inflammatory response IL-1, NF-κB, and TGF-β and stimulates the synthesis of sirtuin-6. KE peptide normalizes the immunological function of human skin fibroblasts during their aging. AED peptide activates the synthesis of sirtuin-1, sirtuin-6, and collagen I in human skin fibroblasts during their replicative aging, which attests to its geroprotective effect.

Key Words: *short peptides; replicative aging; human skin fibroblasts; sirtuins; collagen I*

Involutional changes in the human body begin at the cellular level, when the cells lose their functional activity, ability to proliferate, migrate, and synthesize tissue-specific proteins [9]. The most pronounced age-related changes are observed in the skin cells. The skin is involved in barrier, excretory, immune, and receptor functions of the body as well as in thermoregulation and maintenance of the water-salt metabolism. Age-related skin changes are associated, among other things, with increasing deterioration of the intercellular substance of the dermis due to reduced synthesis of proteins of the intercellular matrix by fibroblasts, on the one hand, and activation of enzymes that destroy it, on the other. Fibroblasts are widely used in the study of repair mechanisms and the analysis of ontogenesis mechanisms in gerontology. They are the main type of dermis cells responsible for the synthesis and secretion of the main components of the dermal matrix, such as collagen, elastin, and glycosaminoglycans [15]. Replicative aging of skin fibroblasts is primarily associated with a decrease in the synthesis of collagen, hyaluronic acid, and other bioactive substances [4].

Skin aging remains a pressing problem of modern gerontocosmetology. The imbalance between the synthesis of the intercellular matrix by fibroblasts and its degradation can be corrected by using peptide geroprotectors [1,3]. A promising methods of slowing down the process of age-related skin changes is the use of short peptides. Short peptides possess geroprotective and immunoprotective properties [2,3,5] and can play an important role in restoring the functions of skin fibroblasts during their replicative aging.

The biological activity of the KE peptide synthesized on the basis of amino acid analysis of thymus polypeptide complex is aimed at enhancing the proliferation and differentiation of thymic epithelial and immune cells [3,5] and stimulation of cellular immunity [5]. The KE peptide exhibited immunomodulatory, anticarcinogenic, antioxidant, and geroprotective effects in *in vitro* and *in vivo* studies [3,5]. In addition, KE peptide can bind to a specific double-stranded DNA sequence and regulate gene expression and cy-

[1]Department of Biogerontology, St. Petersburg Institute of Bioregulation and Gerontology, St. Petersburg, Russia; [2]Department of Therapy, Geriatrics, and Anti-Aging Medicine, Academy of Postgraduate Education, Federal Research Clinical Centre of Federal Medical-Biological Agency of Russia, Moscow, Russia. ***Address for correspondence:*** miayy@yandex.ru. N. S. Linkova

toskeleton protein synthesis, cell proliferation, and metabolism, which explains its high biological activity [11,12].

AED peptide constructed on the basis of a polypeptide complex isolated from cartilage regulated metabolism of the connective tissue in experimental models in animals [1]. Based on these data, we can assume that KE and AED peptides can participate in the regulation of functions of skin fibroblasts.

Signaling molecules that regulate skin cell homeostasis are sirtuin-1 (SIRT1), transcription factor NF-κB, transforming growth factor β (TGF-β), and IL-1. SIRT1 is a multifunctional protein involved in the response to stress, cellular metabolism, and aging through deacetylation of various substrates, including histones and transcription factors. SIRT1 regulates energy homeostasis, cell cycle, apoptosis, inflammatory reactions, and ROS level in the cell [7]. NF-κB is a universal transcription factor that controls gene expression of the immune response, apoptosis, and cell cycle [14]. Impaired synthesis of NF-κB promotes inflammation, autoimmune diseases, and the development of viral infections and cancer. Sirtuin-6 (SIRT6) attenuates the effect of NF-κB, thereby inhibiting replicative cell aging and increases life expectancy: it participates in DNA repair, inhibits pathological signaling cascades associated with IGF-1 and impaired antioxidant system functions, and activates metabolism. SIRT1 inhibits NF-κB activity and COX-2 and iNOS production, thus producing an anti-inflammatory effect [13]. TGF-β is a cytokine that regulates cell growth, differentiation, morphogenesis and apoptosis. TGF-β is a fibroblast growth stimulator [10]. Cytokine IL-1, a mediator of inflammation and immunity, is synthesized primarily by activated macrophages, keratinocytes stimulated by B-cells, and fibroblasts. Collagen I is one of the main structural proteins synthesized by skin fibroblasts, a marker of high functional activity of these cells [6].

Our aim was to study the effects of KE and AED peptides on the expression of sirtuins, collagen, cytokines, and transcription factor NF-κB in human skin fibroblasts during their replicative aging.

MATERIALS AND METHODS

Fibroblasts were derived from the skin around the ear of a female patient (born March 16, 1970) during facelift operation. The patient gave informed consent to the use of surgical material in the research. After harvesting, the skin was treated under sterile conditions with dispase II at (2.4 U/ml) for 18 h at 4°C, then the epidermis was mechanically separated from the dermis. To obtain a cell suspension, the dermis was crushed and placed in a collagenase I solution in M199 medium. The culture medium consisted of M199 medium, 10% fetal bovine serum, 1% L-glutamine, 1.5% HEPES buffering agent, and a solution of penicillin and streptomycin. In 5 days, when the primary culture reached a monolayer, the cells were harvested with trypsin-Versene solution and subcultured in a ratio of 1:3. The cell concentration for zero passage was 50,000 cells per 1 ml medium.

After attaining confluence (3 days), the culture was passaged. Culturing was performed to passage 3 ("young" cultures) or 14 ("old" cultures). After that, the cells were seeded on plates and immunocytochemical staining was performed. By passage 14, the cultures of human skin fibroblasts lost their ability to proliferate and the level of apoptosis in them increased, and therefore this passage was chosen as the model of "old" cells. As inhibition of proliferation and intensification of apoptosis are not characteristic of transformed cells, it can be assumed that the studied cultures did not spontaneously transform. Passage 3 was chosen for the study of "young" cells, because by this time they adapt to growth in culture and can be used in scientific research. The first and second passages are the period of cell adaptation to growth in culture after isolation from the tissue. The specified technique was developed by the authors on the basis of their own experience in the study of cell aging in culture and analysis of published reports on this subject.

Cells of passages 3 and 14 were divided into 3 groups that were further cultured without peptides (control) or in the presence of 20 ng/ml KE peptide or 20 ng/ml AED peptide. Peptide concentration of 20 ng/ml was chosen, because in previous studies, this concentration of other short peptides turned out to be most effective for skin fibroblasts [1]. Then, immunocytochemical staining of the cultures was carried out. The cells were permeabilized with 0.1% Triton X-100 (BioloT) in PBS, incubated in 1% BSA in PBS (pH 7.5) for 30 min to block non-specific binding, and incubated for 60 min with primary antibodies to collagen I (1:100, Abcam), SIRT1 (1:100, Abcam), SIRT6 (1:200, Abcam), IL-1 (1:75, Abcam), NF-κB (1:100, Abcam), and TGF-β (1:75, Abcam). These signaling molecules play the leading role in the processes of cell aging and maintenance of functional activity of skin fibroblasts. Then, the cells were incubated with secondary antibodies conjugated with fluorochrome Alexa Fluor 488 (1:1000; Abcam) or Alexa Fluor 647 (1:1000, Abcam) for 30 min at room temperature in the dark; green or red fluorescence characterized the expression of the studied markers. Cell nuclei were poststained with Hoechst 33258 (Sigma) and they fluoresced dark blue. Finally, the preparations were placed under coverslips in a Dako Fluorescent Mounting Medium (Dako). Confocal microscopy of the cells was

performed using an Olympus Fluoview CM FV300-IX70 inverted confocal microscope with apochromatic 606 UPlan lens.

To analyze the obtained results, Video-TesT-Morphology 5.2 software (Russia) was used. In each case, 5 fields of view were analyzed at ×200. The expression area 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 in which the studied marker is expressed.

Statistical data processing included calculation of the arithmetic mean, standard deviation, and confidence interval for each sample and was carried using Statistica 6.0 software (StatSoft, Inc.). The type of distribution was determined using the Shapiro—Wilk test. To test statistical homogeneity of several samples, non-parametric one-way ANOVA (Kruskal—Wallis test) was used. In cases where analysis of variance revealed a significant heterogeneity of several samples, for subsequent identification of heterogeneous groups (by pairwise comparisons), multiple comparison procedures were used using the Mann—Whitney U test. The critical level of reliability of the null hypothesis (the absence of differences) was set at 0.05.

RESULTS

Expression of SIRT1, SIRT6, and collagen I in old cultures was significantly lower than in young cultures (by 1.8, 3.6, and 3.5 times, respectively) (Fig. 1). The obtained data indicate that replicative aging is associated with a decrease in the number of skin fibroblasts with active synthesis of SIRT1, SIRT6, and collagen I.

Peptide KE increased the expression area of collagen I in old fibroblast cultures by 1.8 times ($p<0.05$) in comparison with the control and significantly increased the expression area of SIRT6 in young and old fibroblast cultures by 1.6 and 2.6 times, respectively, in comparison with the control (Fig. 1). Peptide AED increased the expression of SIRT1 and collagen I in old cultures by 2 and 2.7 times ($p<0.05$), respectively, and the expression of SIRT6 by 11.5 times in comparison with the control values (Fig. 1). In young cultures, addition of peptide AED significantly increased the expression of SIRT1, SIRT6, and collagen I by 2.4, 1.7, and 2.3 times, respectively, in comparison with the control levels.

The expression of cytokines IL-1, TGF-β, and transcription factor NF-κB in "old" cultures was significantly higher than in young cultures (Fig. 2). It is known that an increase in the concentration of cytokines in the blood in old and senescent age is an adaptive reaction aimed at activation of the immune system [2]. Activity of NF-κB increases with age, which leads to chronic inflammation, oxidative stress, and degenerative diseases.

Peptide KE significantly decreased the expression of IL-1, NF-κB, and TGF-β in old fibroblast cultures by 1.6, 1.8, and 2 times, respectively, in comparison with the corresponding levels in the control (Fig. 2). Peptide AED did not change the expression of these proteins in old and young cultures of human skin fibroblasts.

In a previous study, peptides KE and AED showed geroprotective effects in rat skin fibroblasts in a model of replicative aging. Both peptides stimulated the expression of Ki-67 proliferation marker and CD98hc protein that characterizes functional activity of fibro-

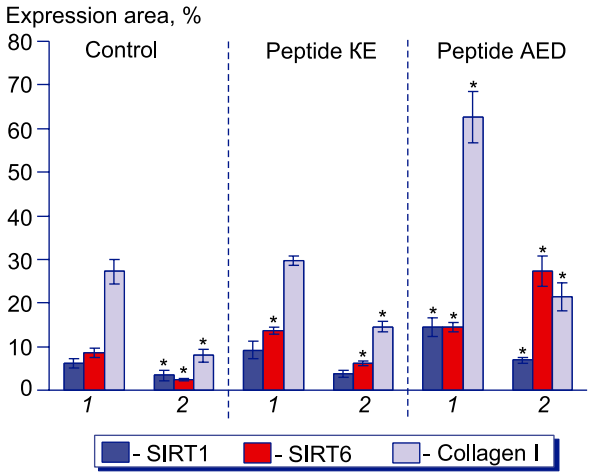

Fig. 1. Effect of peptides on the expression area of SIRT1, SIRT6, and collagen I during replicative aging of skin fibroblasts. *1*) Young culture, *2*) old culture. *$p<0.05$ in comparison with the corresponding parameter in the control.

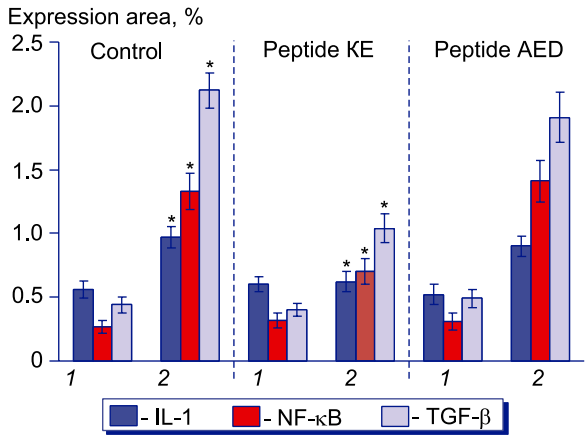

Fig. 2. Effect of peptides on the expression area of IL-1, NF-κB, and TGF-β during replicative aging of skin fibroblasts. *1*) Young culture, *2*) old culture. *$p<0.05$ in comparison with the corresponding parameter in the control.

blasts during cell aging. Peptides KE and AED in rat skin cultures reduced the synthesis of MMP-9 enzyme involved in biodegradation of collagen. Moreover, peptide AED, but not peptide KE reduced the expression of apoptosis marker caspase-3 [1].

In this study performed on human skin fibroblasts during their replicative aging, the difference between the effect of peptides KE and AED was even more pronounced, probably due to species specificity of skin fibroblasts. Peptide KE reduced the synthesis of immune proteins, inflammatory response stimulators IL-1, NF-κB, and TGF-β and stimulated the synthesis of SIRT6. As mild inflammatory process (inflammaging) develops in all body tissues during aging [8], it can be assumed that peptide KE normalizes the immune function of human skin fibroblasts during their replicative aging. These results are consistent with previously identified *in vivo* and *in vitro* normalization of the functions of the immune system cells under the action of peptide KE [3,6,11,12]. Peptide AED activated the synthesis of SIRT1, SIRT6, and collagen I in human skin fibroblasts during their "aging". It is known that oxidative stress developing with age contributes to suppression of the synthesis of proteins-regulators of cell functions, activation of apoptosis, disruption of the structure of fibroblast cytoskeleton, and re-modeling of the intercellular matrix. The geroprotective peptide AED eliminates age-related changes by activating the synthesis of sirtuins and collagen I, thereby contributing to the normalization of fibroblast function. Moreover, peptide KE stimulated the expression of SIRT6 and directly reduced the synthesis of NF-κB. The effect of peptides mediated by signaling cascades is inferior by its biological significance to their direct effect on the synthesis of certain proteins, markers of aging, and functional activity of skin fibroblasts.

Thus, the influence of dipeptide KE is aimed at reduction of the expression of immune proteins (IL-1, NF-κB, and TGF-β) in human skin fibroblasts, prevention of inflammaging, while tripeptide AED activates the synthesis of collagen I, SIRT1, and SIRT6. It can be hypothesized that peptide AED produces a geroprotective effect. Thus, peptides KE and AED regulate the expression of signaling molecules in skin fibroblasts during aging.

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