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Influence of AEDG and KE Peptides on Mitochondrial Staining and the Expression of Ribosomal Protein L7A with Aging of the Human Pineal Gland and Thymus Cell In Vitro

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Abstract—New molecular targets for the geroprotective activity of AEDG (epitalon) and KE (vilon) peptides were verified via confocal laser scanning microscopy. It was shown that the aging of pineal and thymic cells in vitro led to a decrease in the staining of MitoTracker Red mitochondria and that there is a compensatory increase in the synthesis of L7A ribosomal protein. AEDG peptide increased the area of MitoTracker Red mitochondrial staining by 1.5 times and decreased by 22% the expression of ribosomal protein L7A in cultures of human pineal-gland cells with aging. KE peptide increased the area of MitoTracker Red mitochondrial staining by 1.5 times and decreased by 15% the expression of ribosomal protein L7A in cultures of human thymic cells with aging. The area of MitoTracker Red mitochondrial staining decreased and the compensatory expression of L7A ribosomal protein increased with aging of the pineal gland and thymic cells. Presumably, AEDG and KE peptides have a tissue-specific effect that normalizes the functions of mitochondria and ribosomes of pinealocytes and thymocytes.

Keywords: short peptides, pineal gland, thymus, aging, mitochondria, ribosomes

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INTRODUCTION

Modern concepts allow us to consider aging as a process of chronic, weak inflammation (inflammaging) [22]. This process is observed in elderly people and is expressed by an increase in the level of inflammatory mediators, such as the blood contents of CRP, IL-6, and fibrinogen [22]. The trend towards an increase in the average life expectancy of the population emphasizes the need for a deep study of the mechanisms associated with age-related decline in the functioning of the immune system. It is known that T lymphocytes are most susceptible to age-related changes. Involution of the bone marrow and thymic microenvironment leads to a decrease in the number of T lymphocytes and extinction of the function of the adaptive immune system [18, 19].

In recent decades, the role of mitochondria in the aging process has been actively discussed.

Mitochondria consist of separate compartments. The matrix is surrounded by an inner mitochondrial membrane, which is separated by an intermembrane space from the outer membrane. The outer mitochondrial membrane contains the proteins Tom70 or Tom20, which have a transport function. Both proteins are key for mitochondrial identification. The inner mitochondrial membrane has a membrane potential that triggers ATP synthesis. Thus, the functioning of the inner membrane, namely, the maintenance of the potential, serves as an important criterion for the assessment of the performance of a given organoid and of aging processes [24].

The detailed study of signaling intracellular communications made it possible to identify a promising marker, L7A, which is used to visualize the 60S ribosomal subunit. Protein L7A is part of this subunit. It was found that its expression is capable of reflecting the nature of the development of tumor growth and aging processes [25, 27–29].

ROLE OF MITOCHONDRIA IN CELL AGING. MITOTRACKER RED AS A MARKER OF THE STATE OF MITOCHONDRIAL FUNCTIONING DURING AGING

The development of fluorescent dyes has revolutionized the study of organelles under various experimental conditions, since these dyes can be directly incorporated into organelles, which allows them to be visualized under a microscope. MitoTracker probes are one of the fluorescent dyes used to label mitochondria. MitoTracker is a cationic fluorophore that accumulates in mitochondria. Unlike TMRM and rhodamine 123, MitoTracker dye has a reactive chloromethyl group, which forms a covalent bond with the thiols of mitochondrial proteins and peptides [20].

An important advantage of the MitoTracker probe is its ability to stain live and fixed cells. Organoids retain the MitoTracker dye after the loss of their membrane potential [9]. This property of MitoTracker made it possible to study the morphology of mitochondria, their number, and their bioenergetics [24]. Since the hallmark of the early stages of apoptosis is the destruction of mitochondria, including changes in membrane and redox potential, MitoTracker Red is specifically designed to analyze the potential of mitochondrial membranes in living cells via flow cytometry and confocal microscopy [9].

In addition, T cells undergo metabolic reprogramming during their differentiation [26]. It was shown that resting T cells, such as naive T lymphocytes and memory cells, primarily use highly efficient oxidative phosphorylation, while catabolic reactions are represented in effector T lymphocytes by aerobic glycolysis. Thus, the modulation of metabolic pathways and T cell requirements depends on specific T-cell subpopulations. Pyruvate forms in the presence of oxygen. It is completely oxidized in mitochondria to provide energy in many types of cells [17]. In T cells, a significant part of the glucose is not oxidized but is fermented into lactate by lactate dehydrogenase. Despite the presence of oxygen, this process is called aerobic glycolysis, or Warburg metabolism. Although this metabolism is considered energetically inefficient, the glycolysis rate is 10–100 times higher than the oxidation of glucose by mitochondria, which gives equivalent amounts of ATP [13]. An additional benefit of Warburg metabolism lies in the pathways that are branch points of glycolysis (e.g., the pentose-phosphate pathway). A high production of ATP, which is synthesized via enhanced glycolysis, induces T-cell proliferation.

In addition to energy metabolism, mitochondria are involved in the activation of T cells via the influence of secondary messengers. Upon the activation of T cells, mitochondria are localized at the immune synapse and at the locus where they regulate the transport of calcium ions [19]. Upon the penetration of the cell by calcium ions, ROS production increases during the functioning of complex III of the respiratory chain. This leads to an increase in the work of the nuclear factor of activated T cells (NFAT) and the subsequent synthesis of IL-2. A decrease in the transport of calcium ions is observed in aging T cells. This may be partly due to a deficiency in the regulation of the metabolism of mitochondrial calcium [17]. This leads to disruptions in the functioning of the immune synapse and leads to a decrease in the signaling and activation of T cells.

RIBOSOME FUNCTIONING WITH AGING. RIBOSOMAL PROTEIN L7A

The human ribosomal protein L7A is highly conserved. It contains 266 amino-acid residues and has a molecular weight of 30 kDa. L7A consists of three different nuclear localization domains: amino-acid residues 23–51 (domain I), amino-acid residues 52–100 (domain II), and amino-acid residues 101–220 (domain III) [8]. It was established that there is a relationship between myokine (Mnt) and nucleolar function and aging in Drosophila. Overexpression of *Mnt* suppresses the expression of genes encoding nucleolar proteins, which, in turn, decrease rRNA levels. The knockdown of proteins involved in rRNA synthesis and ribosome biogenesis prolongs the average lifespan [25].

The mechanisms of translation regulation, which can affect the lifespan of organisms, have been described [6, 10]. The lifespan of organisms can increase with a decrease in the intensity of translation processes, which can be achieved via action on ribosomal proteins [23, 27]. Thus, the study of the expression of proteins that make up the ribosome subunits will contribute to a better understanding of the mechanisms underlying aging.

Ribosomal proteins are a diverse group of proteins that, in addition to their general participation in ribosome assembly, may perform other functions. One of the ribosomal proteins associated with aging is the L7A protein. This protein is part of the large ribosome subunit (60S) and plays a critical role in their stabilization by binding to mRNA. This protein can participate in cell growth and differentiation by interacting with the human thyroid hormone THR receptor and the retinoic acid RAR receptor [11].

The dipeptide vilon stimulates the cellular immunity and nonspecific resistance of the organism and also has a stimulating effect on macrophages and neutrophils [1, 2]. The mechanism of action of KE peptide is associated with its activating effect on T cells, which contributes to the recognition of the complex of the peptide epitope with the molecule of the major histocompatibility complex located on the macrophage surface. The application of vilon had a depressing effect on spontaneous carcinogenesis in female CBA mice. It was characterized by a 1.5-fold decrease in the incidence of tumors [1]. The immunomodula-

tory, anticarcinogenic, antioxidant, and geroprotective effects of KE peptide in in vitro and in vivo studies may be related to its ability to interact with a specific TCGA DNA sequence and to regulate the expression of genes involved in these processes [15].

The tetrapeptide epitalon in in vivo and in vitro studies has antioxidant activity, regulates the functions of the pineal gland and melatonin synthesis, stimulates neuronal differentiation , has immunostimulating properties, reduces the risk of the development of tumor diseases, regulates the functional activity of the neuroimmunoendocrine system, helps to increase the telomere length in normal fibroblasts and blood and to overcome the Hayflick limit [3, 5, 7, 14, 21]. The goal of the work was to study the effect of the peptides vilon (KE) and epitalon (AEDG) on the expression of the ribosome protein L7A and the area of mitochondrial staining with MitoTracker Red in cell cultures of the human thymus and pineal gland.

EXPERIMENTAL

The study was carried out on cultures of human thymocytes and pinealocytes. Samples of thymus tissue (1–2 mm^3) were obtained during surgery to eliminate congenital heart defects in a 3.5-year-old child, since part of the thymus is removed during the provision of surgical access to the heart.

Pineal-gland tissue was obtained from a 25-year-old man during an operation to remove a pinealoma. In both cases, informed consent was obtained from patients or their relatives for the use of the operative material in scientific research.

Immediately after their obtainment, the tissues of the thymus and pineal gland were placed in a transport medium [DMEM medium + 1% antibiotic/antimycotic (Gibco, United States)]. Prior to the isolation of cells, the tissues were washed with Dulbecco's solution without calcium and magnesium ions. Fragments of the thymus and pineal gland were placed in a solution of collagenase I and II in a 1 : 1 ratio (Gibco, United States).

The thymus cells were grown in a CO_2 incubator under standard conditions (5% CO_2, 37°C) in a medium containing 15% FBS (fetal bovine serum), 82.5% RPMI, and 1.5% HEPES buffer with the addition of 0.5 mM L-glutamine. The pineal cells were cultured under the same conditions in a medium containing Neurobasal-A ratio (Gibco, United States), 1% FBS, 0.5 mM L-glutamine, and 2% serum-free supplement B-27 (Gibco, United States). Trypsin-Versene solution was used in a 3 : 1 ratio to reseed cells. The cultures were grown up to the third passage ("young" cultures) and up to the 14th passage (old cultures). By the 14th passage, the human thymus and pineal gland cell cultures lost their ability to proliferate, and the apoptosis level in them increased; therefore, this passage was chosen as a model of replicative cell aging.

All samples of cell cultures were divided into three groups: the first (control) underwent the addition of saline; the second underwent the addition of vilon dipeptide (KE) at a concentration of 100 ng/mL; and the third underwent the addition of epitalon tetrapeptide (AEDG) at a concentration of 100 ng/mL. Saline and the studied peptides were introduced into the cultures at each cell subculture.

The L7A protein was verified via immunofluorescence with primary anti-RPL7A antibodies (rabbit polyclonal, 1 : 100, Abcam, United States). The mitochondria were identified with the Red FM MitoTracker dye (Thermo Fisher Scientific, United States).

The preparations were stained according to the standard technique. An Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Japan) and the Olympus FluoView software, v. 3.1b, were used to analyze the results. In each case, ten visual fields were analyzed at magnification of 400×. The relative expression level (%) was calculated with the Morphology 5.2 program (VideoTest, Russia) to evaluate the results. The expression level was determined as the ratio of the area of immunostained cells to the total area of cells in the field of view and was expressed as a percentage. Statistical processing of the experimental data was carried out with the Statistica 6.0 program according to the Student's *t*-test ($p < 0.05$) with an estimate of the mean value, standard deviation, and confidence interval. The Shapiro–Wilk test was used to analyze the distribution type and to test the null hypothesis. The Kruskal–Wallis test was used to assess the statistical homogeneity of several samples.

RESULTS AND DISCUSSION

Area of Mitochondrial Staining with MitoTracker Red and the Expression of Ribosome Protein L7A in Pineal-Gland Cell Culture with Replicative Aging

There was a statistically significant twofold lower area of staining with MitoTracker Red in the control group of old cultures of pineal-gland cells as compared to young cultures. AEDG peptide increased the MitoTracker Red staining area by 1.5 times in old pineal gland cell cultures as compared to the control group ($p < 0.05$, Fig. 1, 2). It can be assumed that AEDG peptide increases the number of mitochondria in these neuroendocrine cells upon the replicative aging of pinealocytes.

The next stage of the study was to evaluate the expression of the protein L7A ribosomes in the culture of human pinealocytes upon replicative aging. The ribosome protein L7A is a component of the 60S large subunit and plays a critical role in the stabilization of ribosomes by binding to rRNA.

The expression of ribosomal protein L7A in of pineal-gland cells does not change with age. AEDG

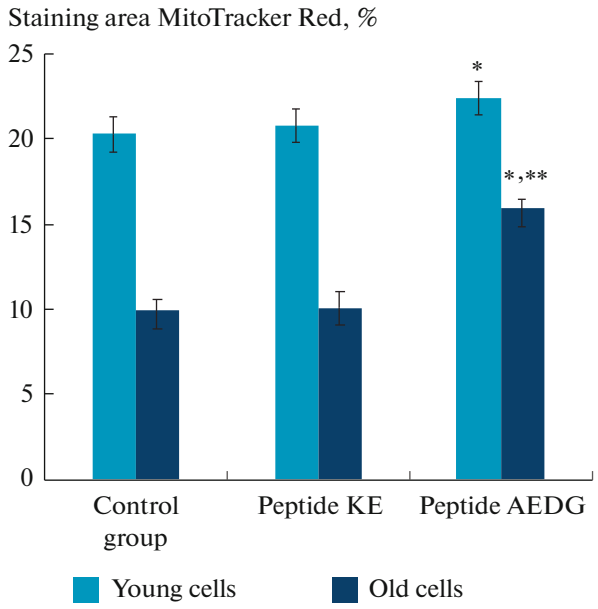

Fig. 1. Area of MitoTracker Red staining in young and old cells of the human pineal gland. * $p < 0.05$ as compared with the control group of young cultures; ** $p < 0.05$ compared with the control group of old cultures.

peptide causes a statistically significant reduction of the L7A protein expression in young cultures by 30% and in old cultures by 22% (Fig. 3).

A number of studies on the expression of the L7A protein in various pathologies have been carried out. An increase in the expression of this ribosomal protein was observed during the adaptation reaction to trauma associated with ischemic disease [12, 16]. There are also studies that have shown an increase in the L7A concentration in prostate carcinoma and colorectal carcinoma [27–29]. The study of age-related disorders of translational processes is an extremely important task to expand our knowledge about aging processes. The lifespan of a living organisms increases with a decrease in the intensity of the replication level due to the effect on the 60S subunit. Thus, the decrease in the expression of the ribosomal protein L7A in pinealocytes during their replicative senescence under the action of AEDG peptide may be one possible mechanism of the geroprotective action of this substance.

Area of Mitochondrial MitoTracker Red Staining and the Expression of Ribosome Protein L7A in a Culture of Thymus Cells upon Replicative Aging

The area of mitochondrial staining with MitoTracker Red in the control of old cultures of thymus cells demonstrated a statistically significant 1.7-fold decrease as compared to young cultures. KE peptide increased the area of MitoTracker Red staining by 1.5 times in old thymus cell cultures as compared to control cultures ($p < 0.05$, Fig. 4). It can be assumed that KE peptide increases the number of mitochondria in immune cells upon the replicative aging of thymocytes.

Studies of lymphoid tissue with aging show the development of a progressively decreasing mitochondrial respiratory function due to a decrease in the mitochondrial membrane potential and impaired

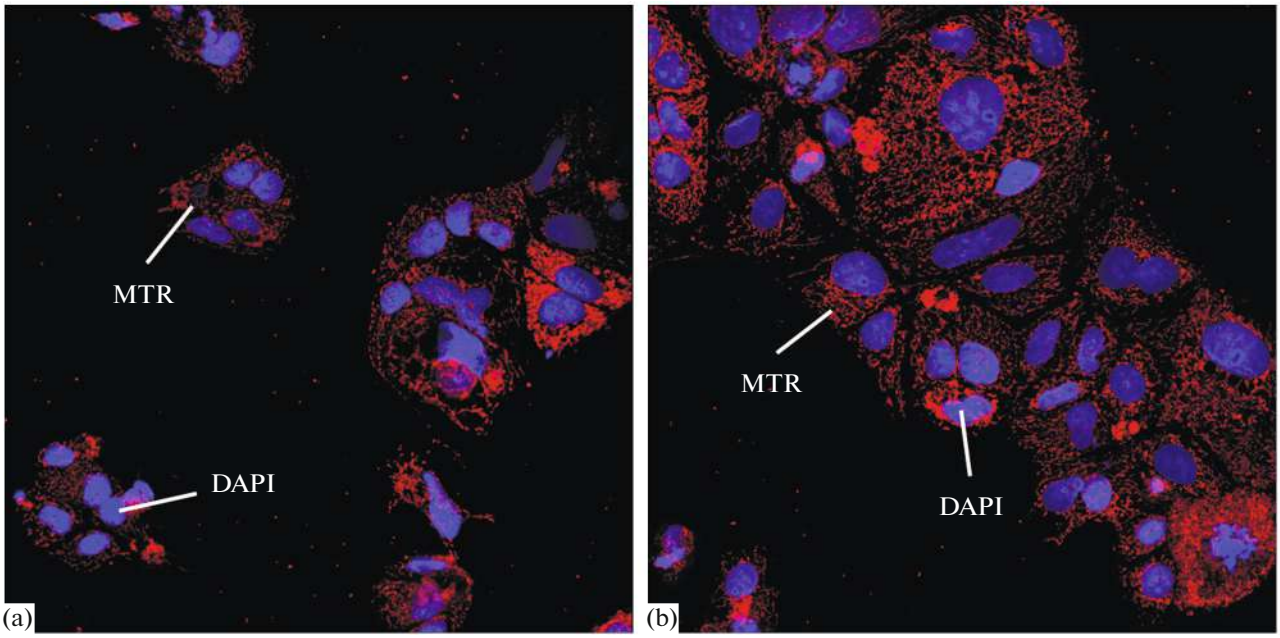

Fig. 2. Area of Mito Tracker Red (MTR) staining in old cells of the human pineal gland, immunofluorescence confocal microscopy, magnification of 400×: (a) control group; (b) AEDG peptide (cell nuclei stained with DAPI).

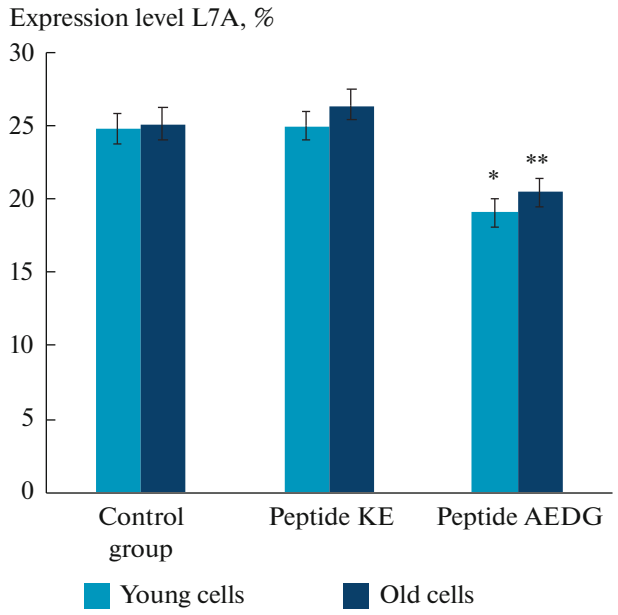

Fig. 3. Expression of L7A ribosome protein in young and old cells of the human pineal gland. * $p < 0.05$ as compared with the control group of young cultures; ** $p < 0.05$ as compared with the control group of old cultures.

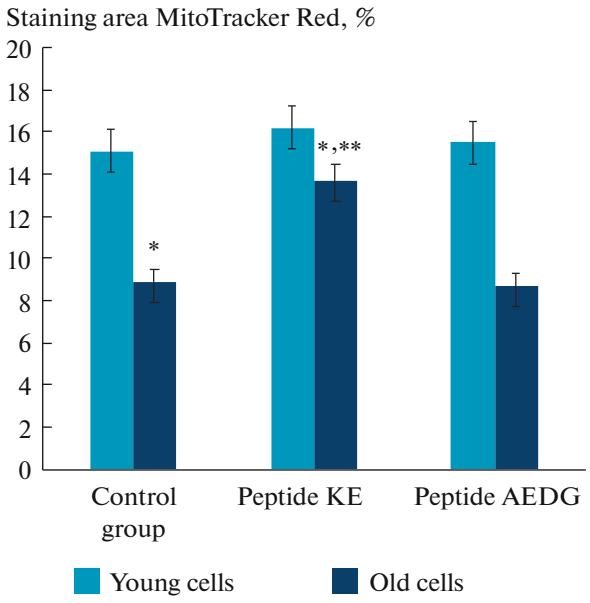

Fig. 4. Area of MitoTracker Red staining in young and old human thymic cells. * $p < 0.05$ as compared with the control group of young cultures; ** $p < 0.05$ as compared with the control group of old cultures.

mitophagy [12]. The production of ATP decreases as a result of the violation of oxidative phosphorylation. This limits the glycolysis, biosynthesis, activation, and proliferation of T cells with aging [10, 17]. Thus, the previously identified immunomodulatory and geroprotective effect of KE peptide on immune cells [2, 4, 5] can be realized via an increase in the functional activity of mitochondria.

With aging, the expression of ribosomal protein L7A in thymus cells demonstrates a statistically significant increase by 12%. KE peptide significantly ($p < 0.05$) reduces the expression of L7A protein by 24% in young cultures and by 15% in old cultures (Fig. 5).

It was established that the nucleolus is involved in the maintenance of stem cells and the regulation of life expectancy, and it plays a role in various cellular functions. In the *Caenorhabditis elegans* model, it was shown that a decrease in the level of expression of ribosomal proteins, which are synthesized in the nucleolus, increases the lifespan of model organisms. In addition, *C. elegans* with knockdown of siRNA NOG-1, which encodes the nucleolar GTPase required for 60S ribosome biogenesis, exhibit an increase in lifespan [20, 25].

Thus, the decrease in the expression of the ribosomal protein L7A in human thymus cells during their "aging" under the action of the KE peptide may be one of the manifestations of the geroprotective effect of this peptide.

CONCLUSIONS

The AEDG and KE peptides have tissue-specific effects via the regulation of the expression of the ribosomal protein L7A and their effect on mitochondria. In cultures of human pineal-gland cells with replicative aging, the expression of the ribosome protein L7A

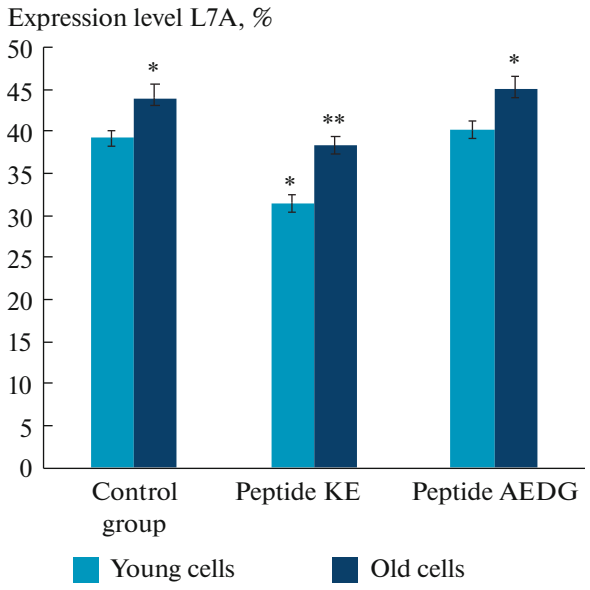

Fig. 5. Expression of protein L7A ribosomes in young and old cells of the human thymus. * $p < 0.05$ as compared with the control group of young cultures; ** $p < 0.05$ as compared with the control group of old cultures.

decreased under the action of AEDG peptide and the area of mitochondrial staining with MitoTracker Red increased as compared to the control.

A similar effect was revealed in cultures of human thymus cells with their "aging" under the action of KE peptide. According to the literature, an increase in mitochondrial function and a decrease in the amount of ribosomal proteins are factors that increase the cellular metabolism and slow cell aging. Thus, we have verified possible new targets for the action of AEDG and KE peptides: the ribosomal protein L7A and the mitochondria. This is important for an understanding of the fundamental principles of geroprotection.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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