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Epigenetic Aspects of Peptidergic Regulation of Vascular Endothelial Cell Proliferation in Aging

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Abstract—Short peptides, vesugen and D-7, stimulated the synthesis of the proliferation-associated protein Ki-67, the expression of which decreased during aging in tissue-specific cell cultures obtained from young and old animals and in dissociated vascular endothelial cell cultures. Molecular docking methods were used to reveal the possibility of binding the vesugen and D-7 peptides to the promoter regions of the *MKI*67 gene, which encodes the Ki-67 protein. Both peptides have contact through the CATC sequence with core promoter 5'-agcctcaaccatcaggaaaacaagagt-3', which is located in the *MKI*67 gene (ENSG00000148773) from −14 to +12 bp relative to the transcriptional initiation site. Thus, the vasoprotective effect of vesugen, which was previously revealed in elder people, can be manifested through epigenetic regulation of the *Ki*-67 gene expression.

Keywords: epigenetics, peptides, proliferation, vascular endothelium, aging, molecular modeling
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INTRODUCTION

Peptide vesugen (Lys–Glu–Asp) having high biological activity against cardiovascular and immune tissues was synthesized at the St. Petersburg Institute of Bioregulation and Gerontology [2, 8, 9]. Vezugen, in combination with conventional treatment of older and elderly patients with atherosclerosis, was more effective in comparison with only standard therapy. Peroral administration of vesugen normalizes sleep, decreases manifestations of cardiac arrhythmia, and reduces the frequency of angina attacks in patients with coronary heart disease. In elderly patients with essential hypertension, the peroral use of vesugen, in combination with hypotensive therapy, led to long-term remission between hypertensive crises and a decrease in the total cholesterol and VLDL content in blood [2]. The combined peroral use of vezugen and neuroprotective peptide pinealon in people aged 20–75 years who work in harmful conditions helped to improve the functions of memory, attention, and thinking, to accelerate perceptual-motor reactions, to increase mental capacity, and to reduce the degree of aging of the central nervous system [1].

It is known that the age involution of the vascular system is associated at the molecular-cellular level with a reduction in the intensity of endothelial cell renewal [17]. It has been found that aging of the vascular endothelium led to a decrease in its proliferative capacity and an increase in the frequency of polyploid cells. The number of polyploid cells in human aorta after 40 years reaches 30% of the total number of endothelial cells. This leads to a local violation of the integrity of the endothelial layer, adhesion of monocytes, and the subsequent development of atherosclerotic vascular lesions [3]. There are experimental data showing that the vasoproptective properties of vesugen is due to its ability to regulate the expression of different signal molecules, including the transcription Ki-67 factor of cell proliferation.

Vasugen stimulated the proliferation of the immune and stem cells of humans and animals [4, 6, 12, 15]. The study of the effect of vesugen on T-helpers with the $CD4^+CD25^+$ phenotype showed stimulation of the expression of the Ki-67 marker of cell proliferation [13]. In a model of accelerated aging, vesugen recovered the structure and increased the proliferative capacity of the cells of the rat duodenum by restoring the functional activity of its vascular component [3]. Peptide D-7 (Asp–Ser) has been recently synthesized, which, due to its conformation, may have biological activity similar to vesugen.

The Ki-67 protein is known to be a key marker in the assessment of proliferative cellular activity and the evaluation of the extent of involution processes in different organs and tissues. Vesugen stimulates the cell proliferation, which is probably due to the activation of gene expression of the Ki-67 proliferation protein. One of the main factors of the *Ki*-67 gene transcription is the Sp-1 protein, which interacts with the core promoter of the *Ki*-67 gene at two binding sites located in ranges from −159 to −145 bp and from −14 to +12 bp relative to the transcriptional initiation site [18]. These

regions are responsible for positive regulation of *Ki*-67 gene expression and are important for maintenance of gene transcription activity. It has previously been suggested that a short peptide penetrates the cell nucleus, site-specifically interacts with the gene promoter regions, and epigenetically regulates gene expression [5, 10, 14]. In addition, the molecular modeling method was used to demonstrate the possibility of tetrapeptide–DNA interaction [7]. In this regard, the ability of vesugen and D-7 to activate the synthesis of the Ki-67 proliferation protein can be due to their involvement in the regulation of gene expression of the Ki-67 protein (*MKI*67).

The goal of the work was a comparative study of the effect of the vesugen and D-7 peptides on expression of the Ki-67 proliferation marker in the cultures of vascular endotheliocytes during their aging and an assessment of the interaction between these peptides and the promoter regions of the *Ki*-67 gene.

EXPERIMENTAL

We used organotypic and dissociated types of cell cultures. Organotypic cultivation of vascular tissue of young (three months) and old (24 months) rats of the Wistar line was carried out in Petri dishes in 3 mL of nutritional medium according to a previously described method [11]. The nutritional medium contained 35% Dulbecco medium, 35% Hanks solution, 25% fetal calf serum, 0.6% glucose, insulin (0.5 E/mL), and gentamicin (100 E/mL). The vascular explants were cultivated for three days in incubator at 37°C under 5% CO_2. Organotypic cell cultures were divided into three groups, i.e., a control group with the addition of physiological solution and two experimental groups with the addition of vesugen and D-7 at a dose of 0.05 ng/mL. Cell proliferation in the organotypic culture was studied by intravital light microscopy. The growth of the explants was evaluated by the area index as the ratio of the total explant area to the initial area (the area of the central zone). The explants were photographed by an MTH-13 camcorder microscope (Alpha-Telecom, series 10, Russia). The area index was calculated by the PhotoM12 program.

The primary dissociated endothelial cell cultures were obtained from aorta fragments of young Wistar rats (3 months) by enzymatic dissociation with collagenase. The nutrition medium for the dissociated vascular cell cultures contained 15% fetal bovine serum, 82.5% DMEM, 1.5% HEPES, *L*-glutamine, and gentamicin. Cells were passaged on the fourth day at 80% confluence. The cultures were cultivated up to either the third or fourteenth passage, which were considered "young" and "old" cell cultures of endotheliocytes, respectively, in accordance with the recommendation of the International Association for Cell Culture research (USA, San Francisco, 2007). To study the dependence of biological activity of the peptides on their concentration, the dissociated endotheliocyte cultures were divided into three groups, i.e. a control group with the addition of physiological solution and two experimental groups with the addition of vesugen and D-7 at a dose of 20 ng/mL.

Immunochemical staining of the cultures of vascular cells was performed with primary monoclonal antibodies to Ki-67 (Novocastra 1 : 50) and secondary antibodies (biotinylated anti mouse immunoglobulins, Novocastra). Permeabilization was carried out using 0.1% Triton X100. The reaction was visualized by horseradish peroxidase and diaminobenzidine (EnVision Detection System, Peroxidase/DAB, Rabbit, Mouse). The results were evaluated by morphometric study with a computer analysis system of microscopic images consisting of a Nikon Eclipse E400 microscope, a Nikon DXM1200 digital camera, and the Videotest Morphology 5.2 software. The relative expression area was calculated as the ratio of the area occupied by immunopositive cells to the total area of the cells within the field of view and expressed in percentage.

To obtain the computer model of the peptide–DNA interaction, we used the ENSEMBL database, no. ENSG00000148773, to analyze the *MKI*67 gene (the gene of the Ki-67 protein) and its promoter regions in the ranges from −159 to −145 bp and from −14 to +12 bp relative to the transcriptional initiation site. We chose the DNA sequences from the found promoter regions and studied the interaction of the peptides with these DNA fragments by the molecular modeling methods.

Molecular modeling of the DNA–peptide complex was performed by the Molecular Operating Environment 2012 program [16]. The optimal relative orientation of the peptide and DNA molecules upon their binding and the formation of the stable complex was calculated by the molecular docking method, with the contact area, the number of hydrogen bonds, and parameters of hydrophobic and electrostatic interactions being taken into account. An Amber 12EHT force field and a GBVI/WSA genetic search algorithm were used. After the model was obtained, the DNA and peptide molecules were protonated at pH 7 and $T = 300$ K. The energy of the DNA–peptide interaction was determined by the estimator S value (kcal/mol), which was calculated by the formula:

$$S \approx c + \alpha\left[\frac{2}{3}(\Delta E_{\text{coul}} + \Delta E_{\text{sol}}) + \Delta E_{\text{vdw}} + \beta\Delta SA_{\text{weighted}}\right],$$

where c is the value of the loss of rotational and translational entropy of the complex; α, β are experimental constants dependent on the force field; E_{coul} is the Coulomb energy value, which is calculated using the system charge when the dielectric constant is 1; E_{sol} is van der Waals contribution to the cooperation energy; SA_{weighted} is contribution of molecular shells to the energy value. We performed ten docking iterations for vesugen and D-7 with each promoter region. The

Table 1. Effect of peptides on expression of proliferative Ki-67 protein in vascular cell cultures during aging

Group	Expression area, %			
	organotypic vascular tissue cultures		dissociated vascular endothelial cell cultures	
	young rats	old rats	young cultures, 3rd passage	old' cultures 14th passage
Control	0.40 ± 0.03	0.11 ± 0.03	1.73 ± 0.23	1.05 ± 0.15
Vesugen	0.52 ± 0.04*	0.18 ± 0.03*	2.17 ± 0.16*	2.02 ± 0.10*
D-7	0.57 ± 0.04*	0.21 ± 0.04*	3.23 ± 0.21*	2.13 ± 0.17*

* $p < 0.05$ compared to corresponding control group.

Table 2. Interaction of peptides with promoter region of the *MKI*67 gene *in silico*

Promoter region	Vesugen		D-7	
	docking *S* estimator (kcal/mol; $n = 10$)	peptide binding site in DNA molecule	docking *S* estimator (kcal/mol; $n = 10$)	peptide binding site in DNA molecule
−159...−145 bp agcagttggcaagct	−7.53 ± 0.25	TGGC; CAAG	−5.50 ± 0.08	GCA
−159...−145 bp agtcttcaaagacag	−6.19 ± 0.11	GTCT	−5.63 ± 0.19	AG, CA
−159...−145 bp ccccgcccacgccct	−7.25 ± 0.00	CGC	−5.29 ± 0.14	CC
−14...+12 bp acgctgcgggcgggcgggcgggcggga	−5.69 ± 0.01	GGCG	−4.84 ± 0.08	CGG
−14...+12 bp agcctcaaccatcaggaaaacaagagt	−7.04 ± 0.01	CATC; GGA	−5.47 ± 0.15	CATC
−14...+12 bp ccaagagcacctaaggaaaaggcccaa	−8.14 ± 0.00	CACC	−6.02 ± 0.00	TAAG

docking solutions were ranked by descending order, from the most energy-effective to the least energetically favorable solutions. Only the most energy-effective solutions were analyzed from each docking solution ($n = 10$).

Statistical analysis of experimental data was performed by the Statistica 6.0 program and included the arithmetic mean calculation, the standard deviations from the mean, and the confidence interval for each group. To analyze the species distribution and to test the null hypothesis, we used the Shapiro–Wilk test. The statistical homogeneity of several groups was evaluated by the nonparametric ANOVA procedure (the Kruskal–Wallis test). Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The action of vesugen and D-7 on the organotypic vascular cell culture of young rats led to a significant increase in the area index by 19 and 22%, respectively, as compared with the control group. The addition of vesugen and D-7 to the organotypic vascular cell culture of old rats increased the area index by 20%. Thus, the peptides stimulated the growth of the organotypic cell vascular culture derived from young and old animals.

The expression area of the Ki-67 proliferation factor in the organotypic vascular cell culture of old rats decreased by 72.5% as compared to cell cultures obtained from young animals. The addition of vesugen and D-7 to the organotypic vascular cell culture of young rats increased the expression of the Ki-67 proliferation factor by 12.5 and 15%, respectively, compared to the control group (Table 1). The action of vesugen and D-7 on the organotypic vascular cell culture of old rats led to the increase in the expression area of Ki-67 by factors of 1.8 and 2.2, respectively (Table 1).

We observed a decrease in the expression area of the Ki-67 marker by a factor of 1.64 in the dissociated endotheliocyte cultures. The addition of vesugen and D-7 to the young cultures led to a significant increase in the expression area of the Ki-67 marker by factors of 1.25 and 1.86, respectively, as compared to the control group. The addition of vesugen and D-7 to the old cultures increased the Ki-67 expression area by factors of 1.97 and 2.02, respectively, as compared to the control group (Table 1).

In order to demonstrate the possibility of epigenetic regulation of gene expression of the Ki-67 protein, we designed the molecular models of the interaction of vesugen and D-7 with the promoter regions of this gene. We searched the gene promoter sequences of the Ki-67 protein in the ENSEMBL database. The *MKI*67 gene is located on the tenth chromosome. We found the promoter sequences of three transcripts of the *MKI*67 gene located in different positions of the chromosome. Each of the studied promoter ranges

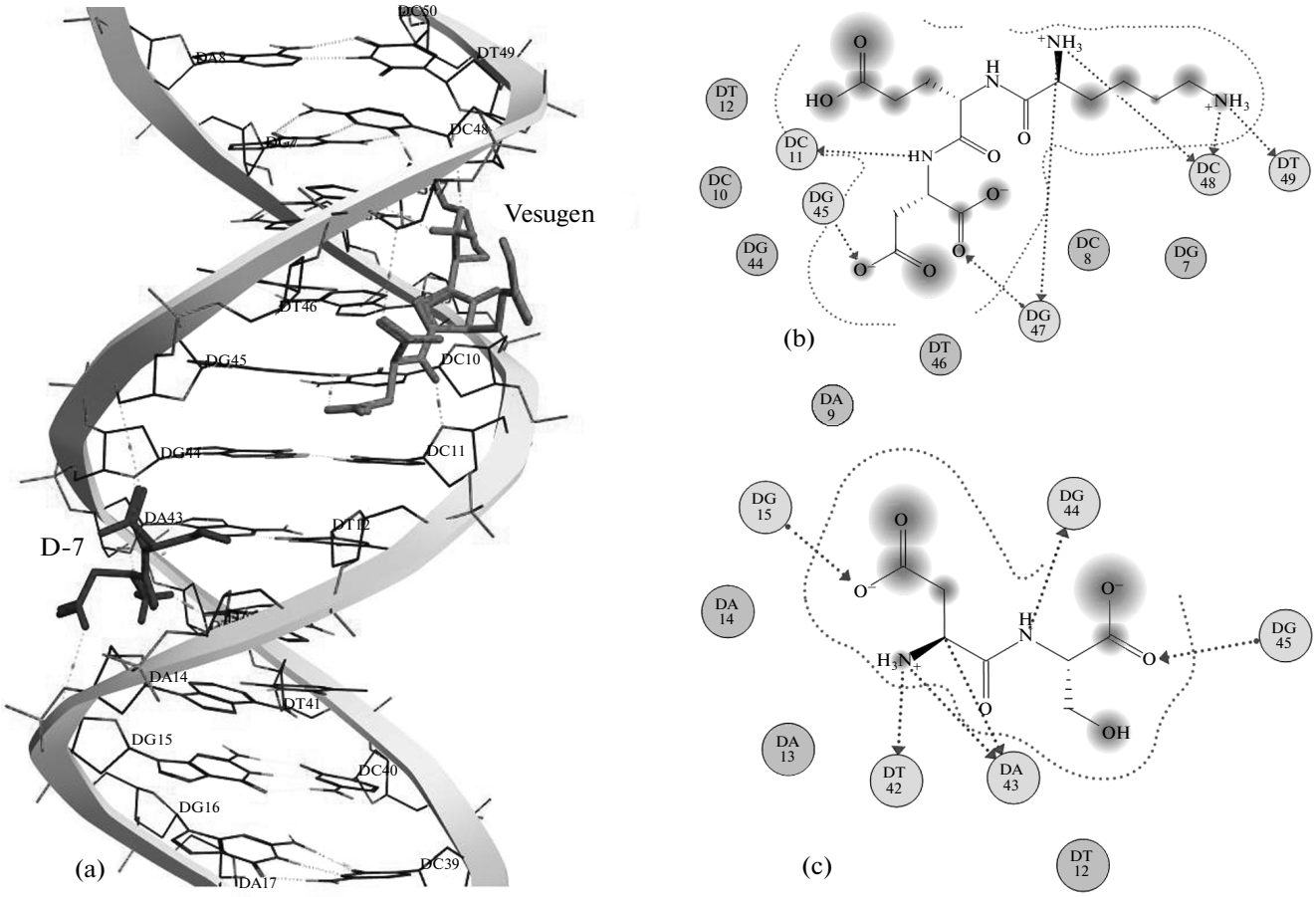

Fig. 1. Interaction of peptides with 5'-ccaagagcacctaaggaaaaggcccaa-3' promoter. Localization of peptides in minor groove of DNA (a); interaction of vesugen with nucleotides in DNA (b); interaction of D-7 with nucleotides in DNA (c). Nucleotides that form donor–acceptor or ionic bonds with peptides are in light grey circles (with arrows); nucleotides at the binding site that do not interact with peptide are in dark grey circles. The dotted arrows show the direction of proton transfer from one atom to another. DC, cytosine; DA, adenine; DG, guanine; DT, thymine. The numbers indicate the sequence numbers of nucleotides in DNA models. Sugar-phosphate backbone is shown as grey helix.

had three different sequences, i.e., 5'-agcagttggcaagct-3', 5'-agtcttcaaagaca-3', and 5'-ccccgcccacgccct-3', in the range from –159 to –145 bp and 5'-acgct-gcgggcgggcgggcgggcggga-3', 5'-agcctcaaccatcag-gaaaacaagagt-3', and 5'-ccaagagcacctaaggaaaaggc-ccaa-3' in the range from –14 to +12 bp relative to the transcriptional initiation site. The docking of vesugen and D-7 was carried out using these sequences. The results are presented in Table 2.

We analyzed the most energetically favorable solutions of the docking of the peptides with DNA regions. Vesugen and D-7 interacted with the DNA molecule mainly in the minor groove. Vesugen was bound with DNA in the major groove only in the case of promoter 5'-acgctgcgggcgggcgggcgggcggga-3' with the GGCG site. However, this interaction was not found among the most energetically favorable solutions, and the interaction energy was 5.72 kcal/mol. Vesugen formed donor–acceptor bonds with three–four DNA base pairs. The peptide was bound to different sites of six promoters; however, the lowest energy was in the case of the interaction with the 5'-ccaagagcacctaaggaaaag-gcccaa-3' promoter at the CACC site (Table 2). The formation energy of this complex was –8.14 kcal/mol (Fig. 1b). Due to the side lysine group, vesugen acted as a proton donor and formed a network of hydrogen bonds with the oxygen atoms O_4 of deoxyriboses and O_2 of cytosine. The carboxyl groups of the side chain of aspartic acid and C-terminal region were, in contrast, proton acceptors and formed hydrogen bonds with the amino group at the second position of guanine (Fig. 1b).

The D-7 peptide interacted with two–three base pairs of DNA and was bound to different sites in the case of all promoters. As in the case of vesugen, the most energetically favorable complex was formed with the 5'-ccaagagcacctaaggaaaaggcccaa-3' promoter but at another site (TAAG), which is located immediately after the binding site of vesugen (Fig. 1a). This peptide formed the hydrogen bonds with the oxygen atoms O'_4

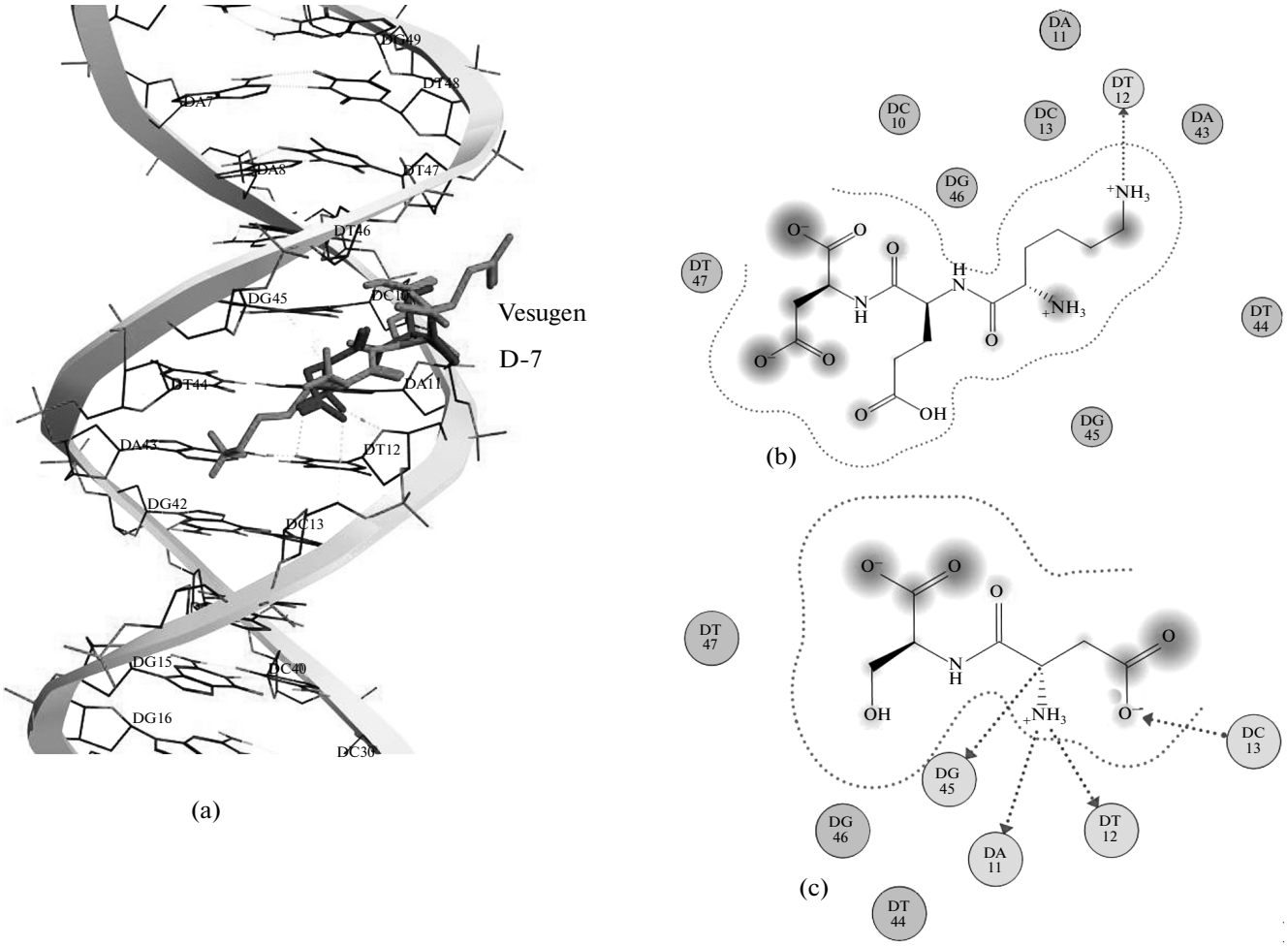

Fig. 2. Interaction of peptides with 5'-agcctcaaccatcaggaaaacaagagt-3' promoter. Localization of peptides in minor groove of DNA (a); interaction of vesugen with nucleotides in DNA (b); interaction of *D*-7 with nucleotides in DNA (c).

of deoxyriboses and O_2 of tymine. The interaction energy was −6.02 kcal/mol. The difference in the binding energy for vesugen and D-7 is most likely due to the additional functional group in vesugen and, thereby, to the increase in the contact area with DNA in the minor groove.

Experiments with organotypic cultures of vascular tissues and dissociated cultures of enothelicyte cells showed that both peptides increased the expression of the Ki-67 proliferation factor, which decreased with vascular cell aging (Table 1). This suggested that vesugen and D-7 have the same binding site in the gene of the Ki-67 protein. This site was found in the 5'-agcctcaaccatcaggaaaacaagagt-3' promoter (Fig. 2a). Both peptides were bound to the CATC site in the minor groove through the hydrogen bond with O_2 of thymine (Figs. 2b, 2c). The binding energy for these peptides differed by 1.5 kcal/mol; *D*-7 formed more hydrogen bonds with DNA than vesugen (Figs. 2b, 2c). Vesugen formed one hydrogen bond between the amino group of the side chain of lysine and the oxygen atom of thymine. D-7 formed four hydrogen bonds between the amino group of the main peptide chain and the atoms of nitrogen N_3 in adenine and oxygen O_2 in thymine and between the carboxyl group of aspartic acid and the CH_2 group of the sugar-phosphate backbone. Along with the hydrogen bonds, the peptides formed the hydrophobic, van-der-Waals, and electrostatic bonds with DNA, and these interactions were also taken into account during evaluation of the energy functions of the peptide-DNA interaction. Therefore, it is not quite right to estimate the profitability of the complex only by the number of the formed hydrogen bonds.

The vasoprotective effect of vesugen was previously revealed in elderly people with different vascular pathologies. The supposed molecular target of the action of vesugen is the promoter region of the *MKI*67 gene, 5'-agcctcaaccatcaggaaaacaagagt-3', which encodes the proliferating Ki-67 protein. This hypothesis is based on data on the increase in the expression of the Ki-67 protein in the organotypic and dissociated vascular endothelial cell cultures during aging. In addition, it was found that D-7 had a similar effect in

the endotheliocyte cultures. The possibility of the epigenetic regulation of various genes expression was shown earlier in experiments with other short peptides (Lys–Glu, Ala–Glu–Asp–Glu, Lys–Trp–Lys–Lys) [5, 8]. Similarly, the supposed mechanism of the action of vesugen and D-7 was their binding with the promoter regions of the *MKI*67 gene, which are responsible for the positive activation of its transcription. We proposed models of the complex formation between the studied peptides and different binding sites of the *MKI*67 gene and calculated the values of the formation energies of the formed complexes. It was found that vesugen formed energetically more favorable complexes with the DNA molecule than the D-7 peptide. This is most likely due to the presence of the additional functional group in vesugen. Vesugen and D-7 were bound to the promoter of the *MKI*67 gene (5'-agcctcaaccatcaggaaaacaagagt-3') at the same CATC site. The interaction of the peptides with this site probably caused the similar effect of the peptides on the enhancement of the proliferative Ki-67 protein expression.

CONCLUSIONS

Thus, the vasoprotective effect of vesugen observed in elderly people with vascular pathology is probably due to its capacity for epigenetic regulation of the *MKI*67 gene expression, which encodes the proliferative Ki-67 protein. Moreover, the other short peptide, D-7, has a similar effect, according to the molecular modeling and the cell culture studies, and can also be considered a potential vasoprotective agent for age-related pathology of the cardiovascular system.

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