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Molecular Aspects of Anti-Atherosclerotic Effects of Short Peptides

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We studied molecular mechanisms of the vasoprotective effects of tripeptide T-38 and dipeptide RR-1. Short peptides T-38 and the RR-1 activate the processes of cell renewal in organotypic and dissociated cultures of vascular cells during aging by increasing the expression of Ki-67 and reducing the synthesis of p53 protein. T-38 and RR-1 reduce the synthesis of E-selectin, adhesion molecule involved in the formation of atherosclerotic plaques.

Key Words: *short peptides; vascular endothelium; aging; atherosclerosis*

Vascular pathology is one of leading cause of mortality worldwide. Since 1975, vascular mortality occupies one of the first places in the structure of total mortality among elderly people of the Russian population [13]. Among cardiovascular diseases, coronary heart disease (CHD) is the most important problem. Prevention and treatment of atherosclerosis, the key element in the pathogenesis of CHD, is the major problem of modern medicine [2,11,14].

Atherosclerosis is a chronic pathology of arteries of elastic and musculoelastic types characterized by the appearance of cholesterol depositions (atheromatous plaques) in their intima.

The first stage in plaque formation is characterized by enhanced expression of adhesion molecule E-selectin on the membranes of endothelial cells. This leads to increased adhesion of monocytes to the vascular endothelial cells and their differentiation into foam cells. Foam cells express E-selectins, which leads to LDL adhesion to their membranes and triggers the formation of atherosclerotic plaques. At later terms, neutrophils and platelets adhere to this complex [6,11]. Along with enhanced synthesis of E-selectin by endothelial cells, an important role in the development of atherosclerotic lesions in vessels is played by reduced rate of cell renewal processes.

Adhesion of monocytes and neutrophils to the endothelial surface during inflammation and atherosclerosis development is activated by the same molecules of cell–cell interactions: integrins on the membrane of neutrophils and monocytes, E-selectin on the membrane of endothelial cells, and P-selectin of platelets [4,12].

During aging, the content of polyploid cells in the endothelium increases. In the aorta of humans above 40, their content can attain 30% of the total number of endothelial cells, which reflects the age-related decrease in proliferative capacity and activation of apoptosis. This leads to local disturbances in endothelium integrity and monocyte adhesion followed by the formation of atherosclerotic plaques [2].

The search for substances aimed at adhesion molecules and cell renewal markers will open prospects in creation of new drugs preventing the development of atherosclerotic lesions in blood vessels.

Tripeptide T-38 synthesized at St. Petersburg Institute of Bioregulation and Gerontology, along with a number of regulators neuroimmunoendocrine system [5,10], exhibits high biological activity towards cardiovascular and immune tissues [7]. T-38 regulates proliferation of primary trypsinized embryonic mesenchymal stem cells, KF-1 rat fibroblast strain, and human erythromyelosis K-562 cells [9]. On rat model of accelerated aging, tripeptide T-38 restores

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the structure and stimulated proliferation of duodenal cells due to improvement of function of its vascular component [3].

In patients with cerebral atherosclerosis, peroral treatment with tripeptide T-38 improved general status and reduced emotional lability and anxiety [1]. CHD patients receiving a course of tripeptide T-38 (2 capsules, 100 μg/day for 2 days) reported improvement of heart arrhythmia and decrease in the frequency of heart attacks. The tripeptide contributed to a decrease in total blood cholesterol in comparison with the corresponding parameter before and after conventional therapy. This attests to normalizing effect of the tripeptide T-38 on metabolism in vascular endothelium [7].

Recently synthesized dipeptide RR-1 by its conformation characteristics can also possess biological activity similar to that of tripeptide T-38.

Here we compared the effects of short peptides T-38 and RR-1 on the expression of signal molecules in cultures of vascular cells from animals of different age.

MATERIALS AND METHODS

We used 2 types of cell cultures, organotypic and dissociated. Organotypic cultures retain natural proportions between the cell types in the studied tissue, which is important for evaluation of the physiological effects of bioactive substances [8]. Dissociated primary endothelial cell culture allows studying the effect of the test substance on the main target of CHD development in the vascular tissue. Thus, the combination of these two methods provides more complete assessment of the vasoprotective effect of short peptides.

Organotypic culturing of vascular fragments from young (3 months) and old (24 months) Wistar rats was performed in Petri dishes in 3 ml medium as described previously [8]. The medium contained 35% Eagle's medium, 35% Hanks solution, 25% fetal calf serum, 0.6% glucose, 0.5 U/ml insulin, and 100 U/ml gentamicin. The explants of the vessels were cultured for 3 days in an incubator at 37°C and 5% CO_2. To study the concentration dependence of peptide bioactivity, the organotypic cultures of vascular cells were divided into 3 groups: control (addition of saline) and two experimental (additions of T-38 and RR-1 peptides); the peptides were added in concentrations of 0.01, 0.05, 1.00, 10.00, 20.00, and 100.00 ng/ml (5 cell cultures for each concentration of the peptide). Organotypic cultures of vascular cells for immunocytochemical analysis were divided into 3 groups: control (addition of physiological saline) and two experimental groups (addition of T-38 and RR-1 in a concentration of 0.05 ng/ml). Cell proliferation in the organotypic vascular culture was studied by intrevital light microscopy. For quantitative evaluation of the explant growth, area index was calculated as the ratio of the explant area to the initial area (central zone). The explants were photographed using a video camera for a MTH-13 microscope (Alpha-Telecom, series 10). The area index was calculated using PhotoM 1.2 software.

Primary dissociated culture of endothelial cells was obtained from the aorta fragments of young Wistar rats by enzymatic dissociation with collagenase. The nutrient medium for dissociated cultures of vessels contained 15% fetal calf serum, 82.5% DMEM, 1.5% HEPES, L-glutamine, and gentamicin. The cells were subcultured on day 4 after attaining 80% confluence. The culturing was performed until passage 3. For evaluation of the concentration dependence of peptide bioactivity, the dissociated of endothelial cells were divided into 3 groups: control (addition of saline) and two experimental (additions of T-38 and RR-1 peptides); the peptides were added in concentrations of 0.01, 0.05, 1.00, 10.00, 20.00, and 100.00 ng/ml (5 cell cultures for each concentration of the peptide). The effect of different peptide concentrations on the growth of dissociated cultures of endothelial cells was assessed by the mean doubling time in cell population. For immunocytochemical analysis, dissociated cultures of vascular cells were divided into two experimental groups with addition of T-38 and RR-1, respectively, to the growth medium at each passage in a concentration of 20 mg/ml.

Immunocytochemical staining of vascular cell cultures was performed using primary monoclonal antibodies against Ki-67 (1:50; Novocastra), p53 (1:50; Novocastra), and E-selectin (1:50; Dako). The results of immunocytochemical analysis were evaluated morphometrically using computer-assisted microscopic image analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, and Videotest-Morphology 5.2 software. The relative area of marker expression was calculated as the ratio of the area occupied by immunopositive cells to the total area of cells in the field of view.

Statistical processing of the results included calculation of the arithmetic mean, standard deviation, and confidence interval for each sample (Statistica 6.0 software). Analysis of the type of data distribution and null hypothesis verification were performed using Shapiro–Wilk test. Statistical homogeneity of several samples was evaluated by Kruskal–Wallis test. The differences between the groups were significant at $p<0.05$.

RESULTS

Effect of short peptides on the expression of signal molecules in organotypic cultures of vascular cells.

TABLE 1. Effect of Different Concentrations of Peptides T-38 and RR-1 on Growth Zone Area Index in Organotypic Vascular Cultures

Concentration, pg/ml	Area index, %			
	"young" cultures		"old" cultures	
	RR-1	T-38	RR-1	T-38
0.01	2.0±0.1	3.0±0.2	2.4±0.4	1.5±0.3
0.05	22.0±1.5*	19.0±1.0*	20.0±1.6*	20.0±1.0*
1.00	20.0±1.6*	18.5±1.9*	20.0±2.6*	19.5±1.0*
10.00	15.3±1.5*	14.0±1.5*	12.1±1.3*	14.0±1.6*
20.00	9.0±0.5*	8.5±0.7*	8.0±0.5*	6.0±0.5*
100.00	8.3±0.5*	8.0±0.5*	6.5±0.5*	3.5±0.4

Note. In the control, area index of "young" cultures is 2.6±0.4% and "old" cultures – 1.9±0.3%. *p<0.05 in comparison with the control.

In organotypic cultures of vessels from young and old rats, the area index significantly increased under the effect of dipeptide RR-1 and tripeptide T-38 in concentrations 0.05 and 1.00 ng/ml in comparison with control cultures (Table 1). Thus, the minimum effective concentration of RR-1 and T-38 stimulating the growth of organotypic cultures of young and old vascular cell cultures was the concentration of 0.05 ng/ml. This concentration was used in further studies of vasoprotective properties of the peptides.

Tripeptide T-38 added to organotypic cultures of young rat vascular cells increased the expression of proliferotropic protein Ki-67 by 12.5% in comparison with the control and inhibited the synthesis of apoptotic factor p53 and E-selectin by 19 and 20%, respectively (Table 2). After addition of RR-1 to the cell cultures, the expression area of Ki-67 increased by 15% and that of p53 and E-selectin decreased by 11% and 3 times.

In organotypic cultures of vascular cells from old rats, T-38 increased the area of Ki-67 expression by 1.8 times and reduced the area of p53 and E-selectin by 21 and 33% in comparison with the control. Dipeptide RR-1 increased the area of Ki-67 expression by 2.2 times and reduced the synthesis of p-53 and E-selectin by 7% and 4.5-fold, respectively (Table 2).

TABLE 2. Effect of Peptides T-39 and RR-1 on the Expression of Signal Molecules in Organotypic Vascular Cultures from Young and Old Rats (*M*±*m*)

Group	"Young" cultures			"Old" cultures		
	Ki-67	p53	E-selectin	Ki-67	p53	E-selectin
Control	0.40±0.03	0.61±0.07	0.91±0.06	0.11±0.03	0.83±0.06	0.43±0.04
T-38	0.52±0.04*	0.55±0.04*	0.78±0.04*	0.18±0.03*	0.64±0.04*	0.32±0.05*
RR-1	0.57±0.04*	0.32±0.05*	0.57±0.05*	0.21±0.04*	0.79±0.05	0.13±0.02*

Note. *p<0.05 in comparison with the control.

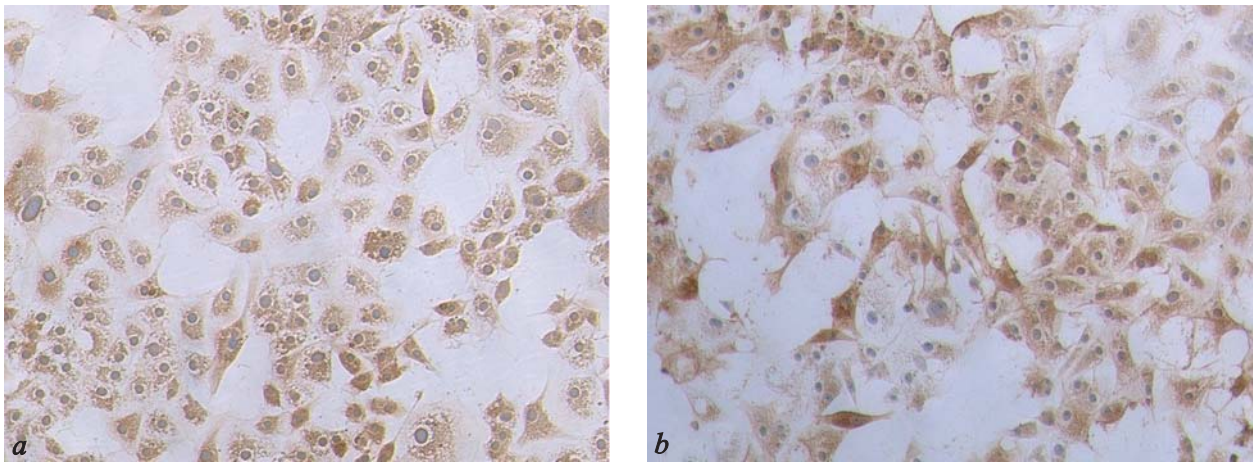

Fig. 1. Expression of proliferotropic protein Ki-67 in dissociated cultures of endothelial cells. Immunocytochemical method, poststaining with hematoxylin, ×100. *a*) Tripeptide T-38, *b*) dipeptide RR-1.

TABLE 3. Effect of Peptides T-38 and RR-1 in Different Concentrations on Mean Doubling Time of Vascular Endothelial Cells in Organotypic Culture

Concentration, pg/ml	Doubling time, h	
	T-38	RR-1
0.01	105.4±5.6	108.4±3.5
0.05	104.0±4.0	103.0±2.5
1.00	101.5±5.5	88.3±3.1*
10.00	92.4±2.0*	65.0±3.4*
20.00	82.4±2.4*	61.0±2.5*
100.00	145.0±7.0*	130.0±5.9*

Note. Mean doubling time in the control was 110.4±5.5 h. *p<0.05 in comparison with the control.

Thus, dipeptide RR-1 to a greater extent and tripeptide T-38 to the lower extent reduced the intensity of apoptosis and expression of E-selectin in organotypic cultures of blood vessels from young and old animals.

Effect of short peptides on the expression of signal molecules in dissociated cultures of vascular cells. Peptides RR-1 and T-38 in concentrations of 10.00 and 20.00 ng/ml reduced the mean doubling time of the cell subpopulation; the maximum effect was produced by the concentration of 20 ng/ml. RR-1 produced similar effect in a concentration of 1.00 ng/ml, whereas T-38 in this concentration was less effective. Peptides in a concentration to 100.00 ng/ml produced opposite effects and increased cell doubling time (Table 3). Shortening of the cell population doubling time attests to an increase in their proliferative capacity, while the decrease in this parameter indicates deceleration

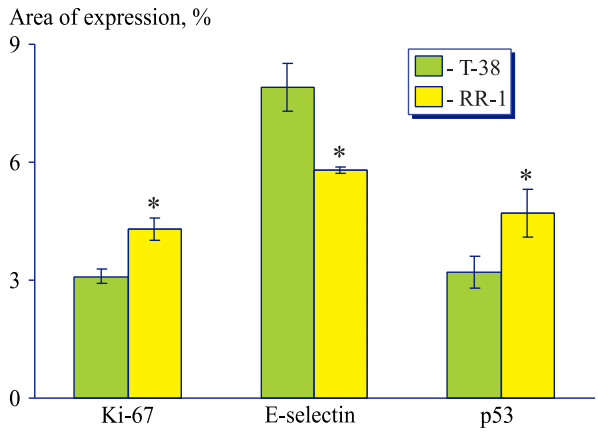

Fig. 2. Effect of peptides T-38 and RR-1 on the expression of signal molecules in dissociated cultures of vascular cells. *p<0.05 in comparison with T-38.

of cell growth. Thus, the maximum stimulating effect on endothelial cell proliferation was produced by the peptides in a concentration of 20.00 ng/ml.

Since peptides T-38 and RR-1 modulate the expression of signaling molecules, markers of functional activity of the endothelium, in organotypic cultures of vascular cells, the functional activity of the endothelial markers, we compared biological activity of these peptides.

Dipeptide RR-1 significantly enhanced the expression of Ki-67 in dissociated culture of endothelial cells by 39% in comparison with tripeptide T-38 (Figs. 1, 2). Under the effect of dipeptide RR-1, the area of E-selectin expression was lower by 27% in comparison with the effect of T-38 (Fig. 2). The effects of RR-1 and T-38 on the expression of proapoptotic protein p53 were similar (Figs. 2, 3).

Thus, the clinical vasoprotective effects of tripeptide T-38 (normalization of lipid metabolism in patients with arterial atherosclerosis and improvement

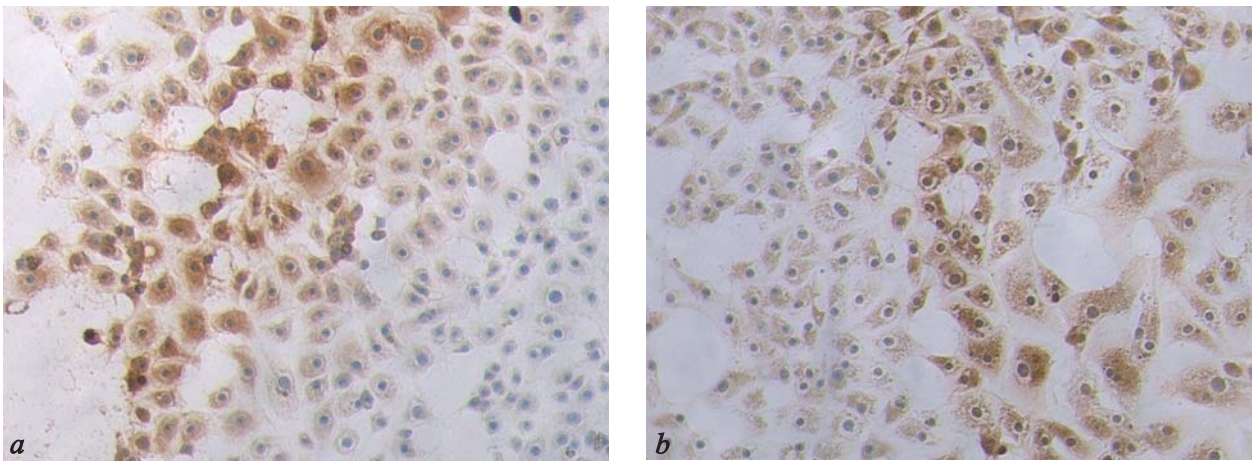

Fig. 3. Expression of proapoptotic protein p53 in dissociated cultures of endothelial cells. Immunocytochemical method, poststaining with hematoxylin, ×100. *a*) Tripeptide T-38, *b*) dipeptide RR-1.

of hemodynamics) is related to its capacity to regulate the synthesis of Ki-67 and p53, the protein markers of cell renewal, and E-selectin, preventing platelet adhesion to the endothelium and formation of atherosclerotic plaques.

Since expression of these signaling molecules decreases during aging of the endothelium, we can hypothesize that T-38 can be useful in prevention of the development of atherosclerosis and CHD in elder individuals.

Moreover, the effects of dipeptide RR-1 and T-38 are mediated via the same molecular mechanism; in some cases, biological activity of RR-1 can surpass that of the tripeptide.

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