ISSN 2079-0570, Advances in Gerontology, 2014, Vol. 4, No. 1, pp. 12–15. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © N.A. Chervyakova, N.S. Lin'kova, N.I. Chalisova, E.A. Kontsevaya, S.V. Trofimova, V.Kh. Khavinson, 2013, published in Uspekhi Gerontologii, 2013, Vol. 26, No. 2, pp. 224–228.

Age-Related Molecular Aspects of Immunomodulating Activity of Peptides in the Spleen

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Received; in final form,

Abstract—In this work, it was shown that short peptides, such as vilon, thymogen, crystagen and R-1, possess different age-associated immunoprotecting properties in the spleen. Both the R-1 and vilon peptides were found to activate T-helper cells. T-helper activation by vilon was based on the inhibition of cellular apoptosis, whereas R-1 acted through positive stimulation of cellular proliferation and differentiation. Thymogen was found to be an activator of B-lymphocytes and acted through the inhibition of apoptosis, as well as by the stimulation of spleen cells proliferation. Crystagen was also found to activate the B-cell immunity system but had no effect on the processes of cellular renewal in the spleen during aging.

Keywords: immune cells, peptides, spleen, aging
DOI: 10.1134/S2079057014010020

INTRODUCTION

An age-associated increase in the incidence of various disorders, including autoimmune, endocrine, infectious, and tumor diseases, is related to the involution of the immune system. The heterogeneous involution of the thymus plays a leading role in aging of the immune system, as a substantial part of the thymus in elderly and senile individuals is replaced with fatty and
1 connective tissues [8]. At the same time, involutive changes in the spleen have not been extensively studied, and it is likely that the functions of the aging thymus can be partially supported by the spleen.

The spleen plays an important role in the immune response. Its main function is the development of
2 macrophages and different types of lymphocytes, including their proliferation and differentiation. The functional activity of the spleen reduces with aging; however, the age-associated changes in its immune function are less pronounced than those of the thymus. The spleen, after the thymus, is the second most important organ of immunogenesis, which is important for both innate and adaptive immunity. Each area of the spleen provides high specificity, mediated by
3 unique interactions between lymphoid and stromal cells. These interactions create a special microenvironment that ensures the implementation of an adequate immune response [6, 14, 15].

Other age-associated changes in the spleen include atrophy of the white and red pulp, resulting in a reduction of the number and size of lymphatic nodes. Age-related atrophy of the spleen is associated with a decrease in the number of macrophages and lympho- 2
cytes in the pulp, leading to reduced functional activity [2].

Several studies have shown that short peptides synthesized at the St. Petersburg Institute of Bioregulation and Gerontology are highly effective towards immune system cells (reviewed in [5]). Amongst these, an immunomodulating activity of vilon, crystagen, thymogen, and thymodepressin was demonstrated.

Vilon is a dipeptide that stimulates T-cell immunity as well as the non-specific resistance of an organism. It has been shown that vilon enhances the expression of lymphocyte activation markers and also stimulates the synthesis of interferons and interleukins [8, 13]. In a model for radiation-stimulated aging, vilon stimulated recovery of the morphological and functional characteristics of the thymus and spleen following γ-irradiation [10]. The effect was mainly related to T-lymphocytes of the thymus and stem cells. Additionally, vilon was shown to increase the average life span of animals, to decrease tumorigenesis, and to inhibit apoptosis [9].

A tripeptide called crystagen has been shown to inhibit proliferation of embryonic mesenchymal stem cells and to enhance the proliferative activity of blood lymphocytes in adults [11]. Crystagen also caused a restoration in the cell content of the main subpopulations of the immune system upon high exercise stress. Moreover, it induced the proliferation of NK cells and

promoted the increased expression of the CD3 immature T-cell marker.

A dipeptide called thymogen is an effective drug for treatment of immunodeficiency disorders, and stimulates the proliferation of T-lymphocytes. In a clinical setting, thymogen administration is used for the treatment of postoperative or radiotherapy-promoted infectious and inflammatory complications [3, 4]. Thymodepressin is an optical and structural isomer of thymogen and is known for its immune inhibiting action. The isomers have a distinct effect on the population of hematopoietic stem cells; thymogen is characterized by its hemostimulating effect, whereas thymodepressin is known for its inhibitory action on hematopoiesis [1].

The data discussed here indicate that the spleen is less affected by age-associated involution than the thymus, and therefore it can be considered a promising subject for investigation of the biological activity of immunomodulating peptides. The goal of the present work was to compare the effects of short peptides on the expression of markers of different immune cell subpopulations in an organotypic spleen cell culture from old animals.

MATERIALS AND METHODS

Organotypic culturing and an immunocytochemical analysis of immunocompetent cell markers were carried out as described [12].

Spleen specimens were obtained from 24-month-old male Wistar rats. The spleen, isolated from freshly killed animals using eye surgery instruments, was placed into a collagen-coated sterile Petri dish (3.5 × 2.5 mm, Jet Biofil) and cut into explants (1 mm^3 fragments, ten fragments per dish). The explants were maintained in 3 mL of a growth medium containing 45% Hank's solution, 45% Eagle's medium, 10% fetal bovine serum, 10 mg/mL glucose, and 0.5 mg/mL gentamicin.

The experiments were carried out using five equal explant groups, among which one was a control group treated with a physiological solution, whereas the explants of the other groups were treated with a peptide (vilon, crystagen, thymogen, or R-1) at a concentration of 0.05 ng/mL. A dipeptide called R-1 is a relatively recently synthesized substance that positively affects the growth of spleen and thymus explants of both young and old rats. The explants used in this work were cultured in a CO_2 incubator at a temperature of 36.7°C in a medium containing 5% CO_2. The explants were grown for 3 days, because it has been shown that this is precisely the amount of time needed for the formation of a growth area consisting of proliferating and migrating fibroblasts, endotheliocytes, and immune cells [12].

To perform an immunocytochemical analysis of the growth area of explants, a culture of spleen cells was fixed using 95% ice-cold ethanol. Immunocytochemical reactions were performed using primary antibodies raised against markers of T-helper cells, CD4 (1:50, Dako), B-lymphocytes, CD20 (1:50, Dako), macrophage maturation, CD68 (1:50, Dako), proliferation, Ki76 (1:150, Vectorlab), apoptosis, p53 (1:50, Dako), and differentiation, CXCL12 (1:200, Vectorlab). A standard single step protocol, including high-temperature antigen retrieval using a citrate buffer (pH 6.0), was employed. Biotinylated anti-mouse immunoglobulins were used as secondary antibodies. The reaction was visualized using a complex of avidin with biotinylated horseradish peroxidase and diaminobenzidine as a substrate (ABC-kit, Dako).

Morphometric analysis of the data was carried out using a computer-assisted microscopic image analysis system consisting of a Nikon Eclipse E400 microscope, a Nikon DXM1200 digital camera, an Intel Pentium 4 based computer, and VidiotestMorphology 5.2 software. In each case ten fields of view were analyzed at a magnification of 100. The area in which a marker was expressed was presented as a percentage of the area occupied by immunopositive cells compared with the total area occupied by cells in the field of view. This value is a widely used morphometric parameter that characterizes the number of cells expressing the marker of interest.

A statistical analysis of the data, including the calculation of the arithmetic mean, standard deviation, and confidence interval for each population, was carried out using Statistica 6.0 software. The Shapiro-Wilk criterion was used to analyze the type of population distribution. To perform a statistical test to determine whether samples from different populations originate from the same distribution, non-parametric procedures for the one-way analysis of variance were used (Kruskal-Wallis criterion).

RESULTS AND DISCUSSION

The expression of immune cell markers, including CD4, CD68, and CD20, as well as markers for the processes of cellular renewal, such as CXCL12, p53, and Ki67, was verified in all control and experimental specimens of the spleen cell culture. The area, in which the markers were expressed, was found to differ in different specimens, confirming previous data relating to the geroprotective properties of the short peptides used in this study.

Effect of Peptides on the Expression of Immunocompetent Cell Markers in the Organotypic Culture of Spleen Cells

The use of vilon and R-1 peptides resulted in a statistically relevant two-fold increase of the area in which the CD4 T-helper marker was expressed, in comparison with the control group. At the same time, thymogen decreased the area in which the CD4 immune cell differentiation marker was expressed by

Effect of short peptides on th expression of immunocompetent cell markers markers of cellular renewal processes in organotypic spleen culture of old rats

Marker	Group				
	control	vilon	crystogen	thymogen	R-1 peptide
*CD*4	0.025 ± 0.005	0.046 ± 0.005*	0.021 ± 0.002	0.01 ± 0.001*	0.047 ± 0.006*
*CD*68	0.017 ± 0.03	0.028 ± 0.05*	0.017 ± 0.004	0.011 ± 0.002*	0.028 ± 0.004*
*CD*20	0.032 ± 0.01	0.014 ± 0.03*	0.048 ± 0.006*	0.08 ± 0.007*	0.023 ± 0.001
*p*53	0.027 ± 0.03	0.011 ± 0.02*	0.030 ± 0.005*	0.012 ± 0.002*	0.026 ± 0.008*
*Ki*67	0.02 ± 0.0006	0.022 ± 0.002	0.02 ± 0.0005	0.04 ± 0.0005*	0.029 ± 0.006*
*CXCL*12	0.038 ± 0.006	0.04 ± 0.0005	0.039 ± 0.003	0.031 ± 0.004	0.085 ± 0.019*

* $p < 0.05$ compared to control group.

2.2-fold, whereas crystagen resulted in no changes (see Table).

The area in which the CD20 B-lymphocyte marker was expressed increased 2.5- and 1.5-fold following application of thymogen and crystagen, respectively, whereas the use of vilon led to a 2.3-fold decrease compared with the control group. The R-1 peptide had no effect on the expression of the B-cell marker (see Table).

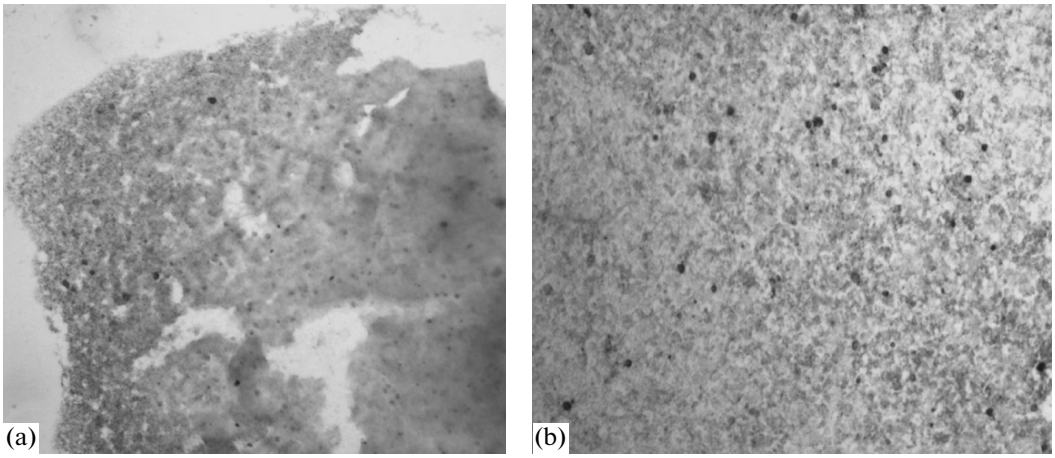
The presence of the R-1 and vilon peptides resulted in a 1.6-fold increase that was statistically significant in the area in which the CD68 macrophage marker was expressed. Thymogen inhibited the expression of this glycoprotein. The area, in which CD68 was expressed, did not change significantly upon the action of crystagen (see Table).

These findings indicate that the R-1 and vilon peptides increase the subpopulation of T-helpers and macrophages in the spleen but have no effect on the number of B-lymphocytes. At the same time, thymogen and crystagen facilitate an increase in the number of B-cells, possess an inhibitory effect on the proliferation (or differentiation) of T-helpers, and show no apparent influence on macrophages in the spleen.

Effect of Peptides on the Expression of Markers of Cellular Renewal in the Organotypic Culture of Spleen Cells

The processes of cellular renewal include three components, such as the differentiation that can be characterized by the CXCL12 chemokine, the proliferation characterized by the Ki65 transcription factor, and finally apoptosis, in which case the p53 transcription factor can be used as a marker. It is well known that equilibrium among these three characteristics of cellular functional activity is shifted to decrease proliferation and differentiation and to enhance apoptosis with aging. Importantly, this effect is observed in all tissues of an organism [7].

Of all the peptides studies here, expression of the CXCL12 cell differentiation marker was increased by use of the R-1 peptide. This peptide resulted in a 2.2-fold increase in the synthesis of the CXCL12



Expression of CXCL12 cell differentiation marker in the organotypic culture of spleen cells from an old rat: a—control group, b—R-1 peptide (immunocytochemical staining, additional eosin staining, ×400).

chemokine marker in comparison with the control group (see table and figure).

The thymogen and R-1 peptides stimulated expression of the Ki67 proliferative protein, resulting in an increase in the area in which this marker is expressed, by 2- and 1.5-fold compared with the control group, respectively. The area in which the Ki67 protein was expressed was unaffected by the action of vilon and crystagen (see table).

The use of vilon and thymogen resulted in a 2.5- and 2.3-fold decrease in the expression of the p53 proapoptotic protein, respectively, whereas the crystagen and R-1 peptides showed no influence on this parameter (see table).

Thus, the peptides that were investigated affect the processes of cellular renewal differently. The R-1 peptide stimulates proliferation and differentiation of spleen cells, thymogen stimulates proliferation and inhibits apoptosis, and vilon exhibits mainly an antiapoptotic effect.

Overall, these findings indicate that, depending on their structure, immunomodulating short peptides can have a different effect on immunocompetent cells and the processes of cellular renewal. For example, both the R-1 and vilon peptides activate the T-cell immunity system; vilon achieves its action by the inhibition of apoptosis, and the R-1 peptide acts through the induction of spleen cell proliferation and differentiation. Thymogen is an activator of B-cell immunity through both the inhibition of apoptosis and stimulation of the proliferative activity of spleen cells. The data obtained in this work indicate that the immune function of the aging spleen can be modulated using peptide-based biological regulators. Moreover, from these data we suggest that further studies of the mechanism of action of peptides toward various types of immunocompetent cells might be promising.

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Translated by S. Khoronenkova

SPELL: 1. involutive, 2. macrophages, 3. stromal