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Research article

Bioregulator Vilon-induced reactivation of chromatin in cultured lymphocytes from old people

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Abstract

The effect of the synthetic peptide bioregulator Vilon on structural and facultative heterochromatin of cultured lymphocytes from old people has been studied. The data obtained indicate that Vilon (a) induces unrolling (deheterochromatinization) of total heterochromatin; (b) activates synthetic processes caused by the reactivation of ribosomal genes as a result of deheterochromatinization of nucleolus organizer regions; (c) releases the genes repressed due to the condensation of euchromatic regions forming facultative heterochromatin; (d) does not induce decondensation of pericentromeric structural heterochromatin. Our results indicate that Vilon causes progressive activation (deheterochromatinization) of the facultative heterochromatin with increased aging.

Abbreviations: Ag-positive – silver stained; C-band – band of centromeric heterochromatin; DSM – differential scanning microcalorimetry; NOR – nucleolus organizer region; SCE – sister chromatid exchange

Introduction

Aging is defined as a manifestation of complex changes in genetic processes that lead to the gradual functional disorders giving rise to senile diseases resulting in inevitable death of an organism. Hence, it appears necessary to develop new medical preparations for slowing down 'the biological clock' and preventing senile pathologies. Special interest is paid to peptide bioregulators – a new type of preparations, peptide Vilon being among them, which is successfully applied in gerontological and geriatric practice. As a result of the peptide bioregulator activities, some metabolic changes occur, regulated through the genes in chromatin domains (Khavison et al. 2002).

It is well established that chromatin is composed of distinct functional domains. Heterochromatin includes the constitutive heterochromatin, almost entirely composed of non-coding sequences of satellite DNA (in metaphases the chromosome regions are designated as C bands and are mostly localized at or are adjacent to centromeric regions) and facultative heterochromatin (condensed euchromatic regions) that mainly consists of 'closed' transcribable genes (Cremer et al. 2000; Carvalho et al. 2001).

In support of this suggestion, the established data adduce, testifying that the presence of only

'active genes' is not enough for transcription, but existence of 'active chromatin' is required as well (Lundgren et al. 2000; Claussen et al. 2002). It has been suggested that progressive heterochromatinization – condensation of eu- and heterochromatic regions of chromosomes accompanied by gene inactivation occurs on aging (Lezhava 2001).

According to this view, we considered it expedient to determine whether the system of chromatin domains in cultured lymphocytes from old individuals undergoes changes when exposed to the peptide bioregulator – Vilon. In particular, our aim was to study the variability of the levels of chromatin condensation in total heterochromatin; nucleolus organizer regions (NORs) (reflecting the activity of synthetic processes); structural heterochromatin and facultative heterochromatin.

Material and methods

We studied donor chromosomes in 46 lymphocyte cultures obtained from 27, 76–81 year old healthy individuals and 12 cultures from 8 young individuals (26–35 years of age). The peripheral blood was taken at 10–11 am from the inhabitants of Tbilisi Boarding House for Aged People. Two cultures (intact and Vilon-treated) were set from each individual that allowed us to compare the indices of treated cultures to their own control values. Vilon at a concentration of 0.01 μg/ml was added to the cultures at the onset and left for an entire period of incubation (72 h).

The parameters of chromatin denaturation defined by differential scanning microcalorimetry (DSM) method were studied in unstimulated lymphocytes obtained from 12 young and old donors.

Description of the preparation – Vilon

Vilon-dipeptide (Lys-Glu) was prepared by directed chemical synthesis on the basis of amino acid analysis of the complex preparation of thymus–thymalin (Khavison 2002).

Mutation

The levels of spontaneous and Vilon-induced aberrations have been studied in cultured peripheral blood lymphocytes as described by Lezhava (1999). In total, 1676 metaphases obtained from 8 old and 712 metaphases from 4 young individuals were analyzed.

Differential scanning microcalorimetry

DSM allows us to study conformational changes in total heterochromatin fraction directly inside the cell. The measurements were carried out on DSM with a sensitivity of 10^{-7} cal/s, the temperature range of measurements – 20–150 °C, scanning rate – 35 K/h, measuring vessel volume – 0.3 ml (Cardellini et al. 2000; Lezhava 2001).

Microcalorimetric investigations of tissues, cells and cellular nuclei showed that the process of denaturation is characterized by well-defined heat absorption peaks. The analysis of numerous experimental data on thermal denaturation of the nuclear chromatin and the chromatin in solution showed that membranes, cytoplasmic structures and nuclear proteins denatured within the temperature range 40–70 °C, while the chromatin denatured at about 60, 76, 88, and 105 °C (Cavazza et al. 1991, Cardellini et al. 2000).

The influence of the peptide bioregulator Vilon on chromatin denaturation parameters inside the intact unstimulated lymphocytes obtained from both – young and old donors and Vilon-treated unstimulated lymphocytes of four old donors (12 cultures) incubated for 22 hours has been studied.

Activity of ribosomal genes of acrocentric chromosomes

This was assessed in 440 metaphases from 5 aged individuals (10 cultures) on the basis of Ag-staining intensity and the frequency of acrocentric chromosome associations by the methods described by us (Lezhava 1999). The probability of argentophilic NORs and the frequency of entering satellite associations by acrocentric chromosomes either in intact or in Vilon-treated cultures were tested by comparison of two binomials.

Polymorphism of structural C-heterochromatin

The structural C-heterochromatin has been examined by the method described by Fernandez et al.

(2002). Three hundred metaphases from 10 lymphocyte cultures (five donors) were studied. The system of classification proposed by Patil and Lubs (1977) was used for comparative analysis of C-stained chromosomes in intact and Vilon-treated cells. The C-segments of chromosomes 1, 9 and 16 were compared to the short arm of chromosome 16. According to this system of classification, the results were distributed to five variants: a, b, c, d and e and evaluated by χ^2.

Variability of the facultative heterochromatin

This was evaluated based on the frequency of sister chromatid exchanges (SCEs) (Lezhava 2001). Short-term lymphocyte cultures were used for differential staining of sister chromatids. In total, 2359 exchanges were detected in 300 metaphases obtained from 10 cultures (intact and Vilon-treated) of five old donors. Equal concentrations of 5-bromdeoxyuridin (BrdU – 7.7 μg/ml) were used for all cultures.

Results and discussion

Mutation

In order to define the non-mutagenic dose of Vilon, several concentrations (0.1–0.01 μg/ml) have been tested. As was found, in cultured lymphocytes from young individuals, Vilon at the concentration of 0.01 μg/ml induced 2.0 ± 0.8 chromosome aberrations per cell that did not exceed their own control values 1.7 ± 0.7. For aged individuals, chromosome aberrations per cell equaled 3.60 ± 0.6 (own control – 2.5 ± 0.6), and the effect was not significant ($P > 0.05$). Common chromosome damages were single and paired fragments, chromatid and chromosome exchanges. These types of aberrations were more frequent in individuals aged from 78 to 81 years. Chromosome breaks had a random distribution. The results of this study correspond to the previous data (Lezhava 1999; Tawn and Whitehouse 2001).

Denaturation of total heterochromatin

Figure 1 shows the calorimetric curves corresponding to denaturation processes in unstimulated (a) intact lymphocytes from old donors; (b) Vilon-treated lymphocytes from old donors.

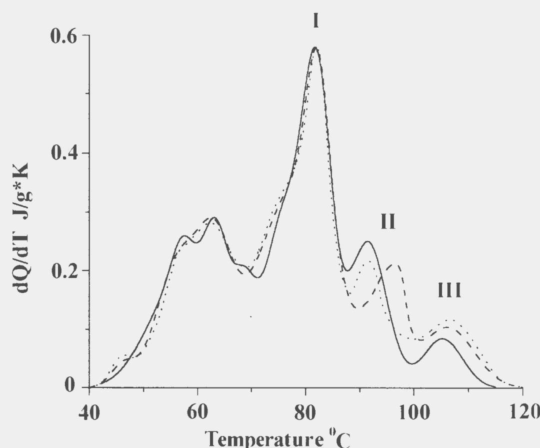

Figure 1. Chromatin heat absorption capacities in lymphocytes cultures (- - -) from old donors; (—) Vilon-treated lymphocyte cultures from old donors; (····) from young donors.

As is seen in Figure 1, the observed heat absorption curves had rather complex profiles. It was already mentioned that chromatin inside the nuclei denatures at a temperature of about 60–105 °C (Cavazza et al. 1991; Cardellini et al. 2000). Therefore, we assumed that endotherms I, II, III corresponded to the denaturation of chromatin inside lymphocytes. The denaturation parameters of these transitions were equal to Td(I) = 82.3 °C; Qd(I) = 33.8 J/g; Td(II) = 95 °C; Qd(II) = 30.2 J/g; Td(III) = 106 °C; Qd(Il) = 28.0 J/g; Q_{inter}(I, II, III) = 92 J/g that coincided well with the melting enthalpy of nuclear chromatin determined earlier in Monaselidze et al. (1981) and Cavazza et al. (1991). The bands within the low-temperature scale with Td peak were equal to 46 ± 1.0; 55 ± 1.0 and 63.0 ± 1.0 °C and should be attributed to the denaturation of membranes, nuclear matrix and cytoplasm structures.

We also showed that the peptide Vilon added to lymphocyte culture caused alterations in profiles of the heat absorption curves (Figure 1). In particular, adding of Vilon to the cultures shifted endotherms II and III to lower temperatures by 2.9 and 1.0 °C, accordingly. Besides, the heat redistribution was observed within stages II and III; at stage II, the heat increases, but at stage III, it decreases. Based on previous data (Cavazza et al. 1991; Cardellini et al. 2000), we suggested that stages I

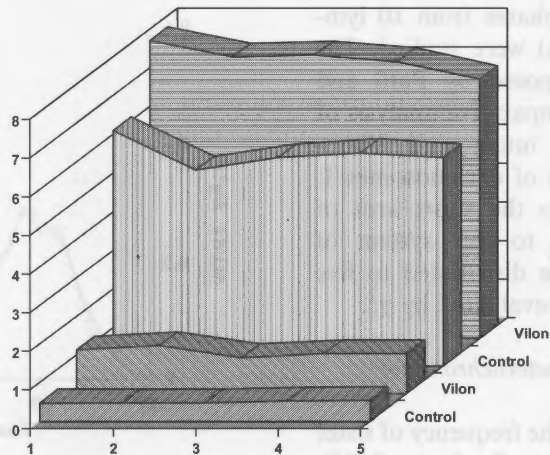

Figure 2. Frequency of acrocentric chromosome associations and Ag-positive NORs in Vilon-treated lymphocyte cultures of aged individuals. (a) Frequency of acrocentric chromosome associations in Vilon-treated lymphocytes and control cultures; (b) frequency of Ag-positive NORs in Vilon-treated lymphocytes and control cultures. On *X* axes are studied individuals.

and II of endotherm transition were related to the unfolding of 10 and 30 nm fibers and stage III – to the unfolding of the loops consisting of 30 nm fibers attached to the nuclear matrix.

The results indicated that the treatment of cells with Vilon induced heat redistribution between stages II and III, which should be attributed to the partial deheterochromatinization–decondensation of the loops up to the 30 nm fibers.

Transcriptional activity of ribosomal genes

The data obtained from the analysis of Ag-positive NORs in intact and treated with Vilon lymphocytes cultures derived from old donors are given in Figures 2a, b. It was shown, that Vilon strongly increased the frequency of Ag-positive NORs in all acrocentric chromosomes – either in involved associations or not – (7.20 per a Vilon-treated cell) in comparison with intact cells – (5.47) ($P < 0.001$). In particular, the frequency of Ag-positive NORs of acrocentric chromosomes, involved in associations corresponded to 2.39 per cell for Vilon-treated culture, which is significantly higher than the corresponding index for intact cultures – 1.10 ($P < 0.001$). The frequency of acrocentric chromosomes involved in associations in the case of the young donors was 1.33 per cell. Vilon also stimulated an increase in associative activity of acrocentric chromosomes. The frequencies of Vilon-treated cells of aged individuals containing associations were significantly higher than those of the intact cultures ($P < 0.001$). It should be noted that Vilon caused an equal increase of all types of acrocentric chromosome associations – DD, DG and GG.

Human ribosomal genes are localized in secondary constrictions (NORs) – in satellite stalks (heterochromatic regions) of acrocentric chromosomes. It was revealed that silver staining (Ag-banding) was inherent only to the NORs intensively functioning at a previous interphase, and the staining intensity corresponds to the intensity of its functioning (Stitou et al. 2000; Lezhava 2001).

The ability of acrocentric chromosomes to connect to form associations is determined by the presence of two chromatid satellite stalks (Lezhava et al. 1999). The associative activity of the strands positively correlates with the intensity of Ag-staining that, in turn, depends on the activity of the ribosomal genes located in NORs. The absence of satellite stalks or silver staining (caused by the condensation of stalks) also testifies to the inactivation of ribosomal genes (Hens et al. 1980; Lezhava and Dvalishvili 1992; Trere 2000).

Our results are in accordance with previous data. In particular, according to some reports, peptide bioregulators and chemicals (Mamaev and Mamaeva 1992; Khavison et al. 2002) induced chromosome decondensation resulting in in-

creased transcriptional activity of nucleolar organizers. It was established that the frequencies of Ag-positive NORs and associations depended on the degree of condensation (heterochromatinization) of satellite strands. The chromosomes of D and G groups with well-defined decondensed satellite stalks show a strong tendency to the formation of associations (Lezhava 2001; Stitou et al. 2002). Our data supports this statement.

An increase in amount and size of Ag-positive NORs, as well as in a number of acrocentric chromosomes involved in associations, in the cultures obtained from old individuals and treated with Vilon, as compared to control values, indicated deheterochromatinization of satellite stalks. This could lead to intensification of protein synthesis processes due to the activation of ribosomal genes in aged individuals.

Heteromorphism of structural C-heterochromatin

The results of comparative analysis of C-segment indices for Vilon are given for three chromosome pairs (1, 9 and 16) in Vilon-added lymphocyte cultures of old individuals (Table 1).

The data reflecting variability of large (c, d and e) and small (a and b) C-segment variant frequencies in Vilon-treated cells were reproducible. It should be noted that the distribution of C-segment variants for chromosome 1, 9 and 16 remained stable and did not differ from those for corresponding intact cells ($\chi^2_4 = 4.99$, $P > 0.05$, $\chi^2_3 = 3.03$, $P > 0.05$ and $\chi^2_2 = 1.07$, $P > 0.05$ – for chromosome 1, 9 and 16 respectively).

It is notable that statistically significant alterations (decrease in size) of C-bands on chromosomes 1 and 9 have been identified in old individuals after induction with peptide bioregulators Livagen (Khavison et al. 2002) and Epitalon (Khavison et al. 2003). The variability of C-heterochromatin on chromosomes 1 and 9 was described for other chemicals (Ott et al. 1998; Haaf and Schmid 2000). The sizes of C-bands on chromosome 16 always remained unchanged.

Thus, according to our data Vilon does not affect structural heterochromatin on chromosomes 1, 9 and 16. Vilon cannot induce changes in the size of C-heterochromatic regions that indicate the stability of structural C-heterochromatin in chromosomes 1, 9 and 16.

Variability of the facultative heterochromatin studied by SCE test

The results of studies on the induction of SCEs by Vilon in lymphocyte cultures of aged individuals

Table 1. Heteromorphism of C-bands on chromosomes 1, 9, 16 in Vilon-treated lymphocytes from old people.

Chromosome	Variants of C-bands	v_i	μ_i	$v_{i\,n}$	$\frac{v_i+\mu_i}{n+m}$	χ^2
1	a	20	17	0.102	0.094	$\chi^2_4 = 4.99$ $P > 0.05$
	b	55	67	0.280	0.312	
	c	69	74	0.352	0.365	
	d	51	37	0.260	0.225	
	e	1	0	0.005	0.002	
9	a	35	25	0.182	0.157	$\chi^2_3 = 3.03$ $P > 0.05$
	b	72	75	0.375	0.386	
	c	67	75	0.349	0.373	
	d	18	13	0.093	0.081	
	e	0	0	0	0	
16	a	62	74	0.329	0.353	$\chi^2_2 = 1.07$ $P > 0.05$
	b	91	91	0.484	0.472	
	c	35	32	0.186	0.174	
	d	0	0	0	0	
	e	0	0	0	0	

v_i – number of a, or b ... or e variants in intact cells; μ_i – number of a, or b ... or e variants in Vilon-treated cells; n – total number of C-band variants in intact cells; m – number of C-band variants in Vilon-treated cells.

Table 2. Effect of Vilon on frequency of SCEs in lymphocytes from old people.

Donors[a]	Age, sex	Number of SCEs (intact cells)		Number of SCEs (Vilon-treated cells)		t	P
		Absolute number	SCE per cell	Absolute number	SCE per cell		
1	77, f	165	5.5 ± 0.43	268	8.9 ± 0.55	4.9	<0.001
2	78, m	175	5.8 ± 0.44	268	8.9 ± 0.55	4.4	<0.001
3	79, f	180	6.0 ± 0.45	371	12.4 ± 0.60	8.5	<0.001
4	80, f	178	5.9 ± 0.44	313	10.4 ± 0.59	6.1	<0.001
5	80, f	171	5.7 ± 0.44	270	9.0 ± 0.55	4.7	<0.001
Total	77–80	869	5.8 ± 0.20[b]	1490	9.9 ± 0.30	11.4	<0.001

[a]Thirty metaphases for each donor were analyzed.
[b]The average control values (young donors) correspond to 7.2 SCEs per cell.

are shown in Table 2. In total, 1490 exchanges were registered in Vilon-treated cultures corresponding on average to 9.9 per cell; for intact cultures of the same individuals, this value was 5.8 SCE/cell ($P < 0.001$).

The analysis showed that Vilon significantly increased SCE counts in A, C, D, E and G chromosomes in comparison with intact cells. No difference between the SCE counts was observed for chromosomes of B and F groups – the effect of Vilon was not significant ($P > 0.05$).

The effect of tested bioregulators Livagen (Khavison et al. 2002), Epitalon (Lezhava et al. 2003) and Vilon on SCE distribution over the groups of chromosomes revealed to be heterogeneous. In particular, Livagen induced increase of SCE counts in chromosomes A, B, C, D, E and G, while Epitalon stimulated SCE rates in chromosomes A, C, D and G. These data indicate that each of the studied bioregulators selectively effect definite chromosomes.

According to the previous data, the exchange processes do not occur in heterochromatin or heterochromatinized chromosome regions (Hawley and Arbel 1993; Lobov and Podgornaya 1999). Therefore, the increased frequency of SCEs under the influence of Vilon indicates decondensation (deheterochromatinization) of the condensed during the aging chromosome regions, followed by the release of the repressed genes located there. Vilon, being able to decondense chromatin, favors release of the genes that were repressed as a result of heterochromatinization of chromosome regions through aging.

Our results, therefore, indicate, that the peptide bioregulator Vilon can induce reactivation (deheterochromatinization) of facultative heterochromatin through its ability to modify heterochromatinized chromosome regions in cultured lymphocytes of aged individuals. No effect of Vilon was revealed in pericentromeric structural heterochromatin (C-bands of chromosomes 1, 9 and 16) in lymphocyte cultures from aged donors.

We suppose that the next step in future studies on aging processes should be directed toward the mapping of condensed (heterochromatinized) regions of chromosomes that were revealed to be activated by the bioregulators.

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