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Effect of synthesized dipeptide L-Leu-Lys on cell proliferation and apoptosis in organotypic tissue culture from rat spleen

N.I.Chalisova

Pavlov Institute of Physiology of Russian Academy of Sciences, Sanct-Petersburg, 199034, Nab.Makarova 6, (RUSSIA)

E-mail: ni-chalisova@mail.ru

ABSTRACT

The effect of first synthesized dipeptide L-Leu-Lys was studied on cell proliferation and apoptosis using the organotypic culture of spleen tissue from 3-mont-old rats. The dipeptide at concentrations in diapason 0.001 -10 ng/ml produce a clearly defined stimulating effect on cell proliferation in these tissues, accompanying by an increase of expression of Ki67 and decrease of P53 expression. One can suppose that this dipeptide with high biological activity create the base for the further experiments in laboratory animals and clinical trials for investigation of their effect on the immune system.

KEYWORDS

Dipeptide;
Leucine;
Lysine;
Spleen;
Tissue culture.

INTRODUCING

The regulation of repair processes in tissues of organism through the stimulation of cell proliferation or its inhibition in apoptosis is accomplished by various regulatory oligopeptides, including dipeptides. L-carnosine (L-Ala-His) is the mostly known regulatory peptide. This dipeptide plays a great role in metabolism, suggesting its specific involvement in intracellular signaling regulation in excitable tissue. It can prevent serotonin-derived melanoid synthesis and neuronal impairment by stress and age-related dysfunctions[1]. The discovery of the ability of carnosine to regulate expression of early response genes broadens the concept about carnosine as a cellular peptide regulator cells[2, 3, 4]. Thymogen (L-Glu-Trp) is known as an immunomodulator[5]. Dipeptide bestim (D-Glu-L-Trp) binds with a high affinity to murine peritoneal macrophages, reducing the adenylate cyclase activity in the membranes of murine macrophages and thymocytes[6].

In our previous study[7] the combinations of 20 encoded amino acids were studied in the organotypic culture of tissues, in which one amino acid has the stimulating, and other the inhibiting effect on the growth zone of spleen explants. I.e. these amino acids produced by their isolated administration the differently directed controlling on the basic cellular processes – proliferation and apoptosis. It was established, that these combinations of amino acids produced always the stimulation of the growth zone, exceeded the stimulation, producing by the isolated stimulating amino acid. The goal of this work is deal with study for the first time of synthesized new dipeptide L-Leu-Lys. This dipeptide was not used neither in laboratory or clinic investigations. The most adequate method for a rapid quantitative determination of the biological effect of preparations being

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examined is the organotypic culturing of tissue fragments. Changes in the amount of cells in the explant growth zone serve as a criterion of the primary screening evaluation of the biological activity of substances.

MATERIAL AND METHODS

All procedures were in accordance with the standard set forth in the eights Edition of guide for the Care and Use of laboratory Animals

Organotypic culturing of tissues was performed as described earlier[5, 7, 8]. Experiments were performed with 1000 explants of fragments of the spleen of mature Wistar rats with body mass 200-250 g. The rats were decapitated by guillotine. The effect of dipeptide on tissue was studied in 17-20 experimental explants and in the same number of control explants. Tissue fragments, prepared under sterile conditions, were separated to smaller pieces (approximately 1 mm^3), which were placed into Petri dishes with collagen support. Nutrient medium consisted of 35% Eagle's medium, 35% Hank's medium, 25% bovine fetal serum. This medium was supplemented with glucose (0.6%), insulin (0.5 U/ml), and gentamycin (100 U/ml). The dipeptide L-Leu-Lys was added to culture medium at different concentration from 0.001 to 10 ng/ml. Petri dishes were incubated at 37^0C for 3 days in a constant-temperature cabinet and then examined using a phase-contrast microscope equipped with a microtelemetric eyepiece (series 10, MTN-13, Alfa-Telekom, Russia). For each explant we determined the area index (AI), which was calculated as the ratio between the total area of explant (together with the zone of migrating cells) and the area of the central zone of explant and expressed in arbitrary units. The AI values of explants were calculated using the PhotoM 1.2 software. The control explants (positive control) grew in dipeptides-free nutrient medium; the experimental explants, in the presence of compound of interest at different concentrations. The negative control was, when the explants of liver tissue developed at the presence of the same dipeptide, as in spleen explants.

The significance of differences in AI values of the control and experimental explants was estimated using Student's *t* test. AI values were expressed in percents; the control AI value was taken as 100%.

For immunocytochemistry there wree used the primary monoclonal antibodies against marker of cell proliferation Ki67 (1: 50, Dako), proapoptotic protein P53 (1:25, Dako) and secondary antibodies – biotinylated antimouse immunoglobulins (Novocastra). The permeabilization was carried out using 0,1 % triton X100. Visualization of the reaction was performed using horseradish peroxidase diaminobenzidine (EnVision Detection System, Peroxidase/DAB, Rabbit, and Mouse). It was used microscope Nikon Eclipse E400, digital camera Nikon DXM1200, and "Videotest Morphology 5.2" software. In each case we analyzed 10 visual fields (x200). The area of expression was estimated as the ratio of immune positive cells area to the total area of cells viewed, and was expressed in percentage.

Data of mass spectrometry of dipeptide LK – M+260.06; data of high performance liquid chromatography – a content of the basic substance by the optic density (λ 230 nm)> 95%; column Waters DeltaPak C-18, 5μ, $100A^0$, 3.9x150 mm; gradient (1-25)% MeCN in 0.1% TFA.

RESULTS

During the first day of culturing, the explants were sprawling over the collagen substrates (Figure 1). A typical pattern of the structural organization of the peripheral zone of explants of different tissues includes the peripheral growth zone and the explant capsule represented by one or two layers of fibroblasts. On the surface, the capsule was covered with mesothelium, which did not form a continuous layer. The cells of mesothelium often move away from one another, round, and lose contact with the basement membrane. As a result, broad gaps are formed at the site of these cells, through which part of cells migrate beyond the explant. The migrating and proliferating cells are represented by macrophages, fibroblasts, and specific cells characteristic of type of tissue – B- and T-lymphocytes. These cells form the peripheral growth zone of explants, changes in which determine the AI. After three days of culturing, if the

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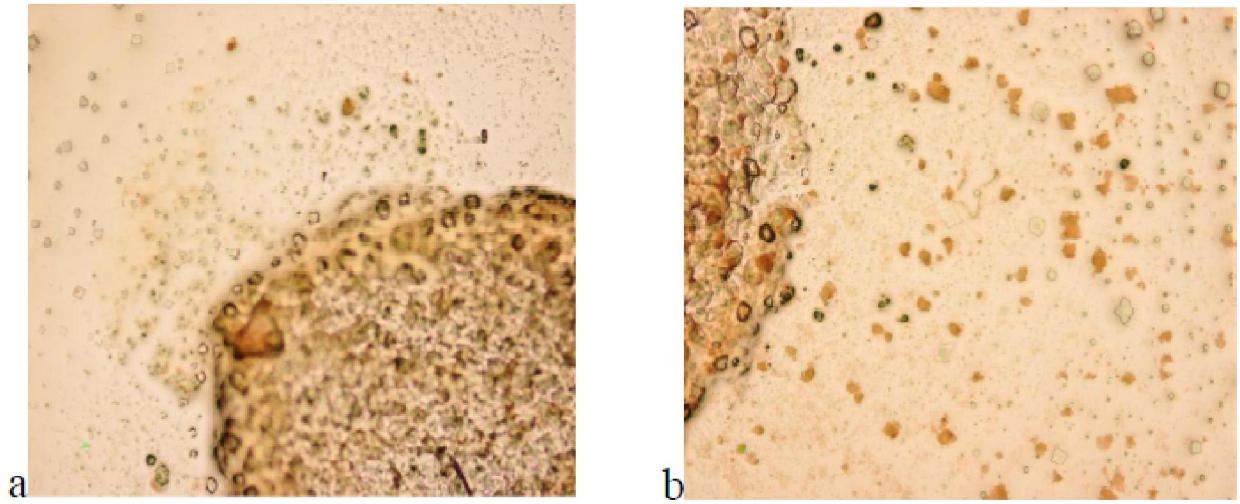

Figure 1 : Expression of Ki67 in the organotypic culture of spleen tissue, x100: a – control, b– by the administration in culture media of dipeptide Leu-Lys in 0,05 ng/ml concentration

TABLE 1 : Effect of dipeptide Leu-Lys on area index (AI), expression of Ki67 and P53 in explants of spleen in organotypic culture

Concentration ng/ml	0.001	0.01	0.05	0.1	1	10
AI, % to control						
	+26±5*	+69±11*	+68±9*	+35±5*	+20±3*	+12±5
expression of Ki67, % to control						
	+19±2*	+48±10*	+37±8*	+20±6*	+15±5	+7±3
expression of P53, % to control						
	-20±4*	-35±11*	-27±6*	-24±5*	-18±7	+6±2

growth zone of the experimental explants increased as a result of stimulation, the AI values of the experimental explants were greater than in the control. If the growth of the experimental explants was suppressed, their AI values were lower than in the control.

The titration indicated the following results**.** In spleen culture of rats, grown in the presence of different concentrations of Leu-Lys (TABLE1) the stimulating the cell proliferation effect was especially pronounced in the case of 0.001 ng/ml (AI increased by 26 ± 5% *(n* = 20, $p < 0.05$), of 0.01 ng/ml (AI increased by 69 ± 11% *(n* = 18, $p < 0.05$), of 0.05 ng/ml (AI increased by 68 ± 9% *(n* = 20, $p < 0.05$), of 0.1 ng/ml (AI increased by 35 ± 5% *(n* = 17, $p < 0.05$), of 1 ng/ml (AI increased by 20 ± 3% *(n* = 19, $p < 0.05$), compared to the control (*n* = 19, 20, 18, 18, 20 according). The concentration of Leu-Lys 10 ng/ml induced a statistically insignificant stimulating effect (AI increased by 12 ± 5% *(n* = 18, $p > 0.05$), compared to the control (*n* = 19). The explants of liver tissue had no reaction to the adding of dipeptide in the nutrient medium and AI of explants were at the control levels.

By the investigation of the spleen explants using immunocytochemistry method it has been shown, that by the effective concentration of Leu-Lys 0.01 ng/ml expression of Ki67 was increased statistically significantly by 48 ± 10% and by the concentration of Leu-Lys 0.05 ng/ml - by 37 ± 8% compared to the control according. At the same time by the concentration of Leu-Lys 0.01 ng/ml expression of P53 was decreased statistically significantly by 35 ± 11% and by the concentration of Leu-Lys 0.05 ng/ml it was decreased by 27 ± 6% compared to the control according (Figure 1).

DISCUSSION

It has been shown previously in organotypic tissue culture[7] the effects of combinations of a standard amino acid at an effective concentration of 0.05

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ng/ml on cell proliferation processes in explants of the spleen, myocardium, pancreas, and cerebral cortex from 24-month old rats. Combinations of two amino acids, one of which stimulated proliferation while the other inhibited it, induced the proliferation of cells in tissue culture. These effects were 8–10% higher than that of stimulating the amino acid. So, in tissue of the spleen lysine stimulated the explant growth by +27±5%, and leucine inhibited it by -32±11%. The combination of these amino acids stimulated the explant growth by +39±7%. Evidently, the supporting of the dynamic balance of the proliferative and apoptotic processes occurs, what is necessary for the tissue development These data may be used to develop new effective dipeptides, which can be used to improve regenerative tissue capabilities.

So, the dipeptide Leu-Lys was synthesized for this study. Thus, we have studied for the first time the effect of new unique dipeptide L-Leu-Lys on the proliferation of cells and apoptosis in an organotypic culture of lymphoid tissue We found that this dipeptide at concentrations in diapason 0.001 -10 ng/ml produce a clearly defined stimulating effect on cell proliferation in a mature spleen tissue of rats. An increase of expression of Ki67 and decrease of P53 expression by the dipeptide action confirms, that their effect is due not only to cell migration, but to increase of cell proliferation and inhibition of apoptosis processes. One can suppose that this dipeptide can unidirectionally regulate cell proliferation in lymphoid tissue.

The preferences of dipeptide are these, that it can pass from the digestive tract without hydrolysis in the blood and so can will used perorally[9, 10]. The dipeptide is not toxic and not immunogenic, for it is constructed from natural amino acids. The fact is of interest, that the effective concentrations for dipeptide in our experiments were in diapason 0.001 -10 ng/ml, i.e., it had the order of magnitude of 10^{-12} M. At lower or higher concentrations (up to 20 ng/ml), the AI values were either statistically insignificant or close to control values, This effect may be associated with the effect of ultra low doses[11, 12]. The effect of ultra low doses is important for the clinic use for the prevention of the allergic reactions.

One can suppose that these new dipeptides with high biological activity will have, like the known peptides carnosine, thymogen, effect on the immune system of organism. The data obtained about the stimulating proliferation activity of dipeptide Leu –Lys at cellular level in culture of lymphoid tissue create the base for its further studies in the experimental animals and by the clinical trials for investigating of their effect on the immune system and circulation at the level of organism.

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