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Protective Effect of Tripeptide in the Presence of Cyclophosphamide on the Growth of Cultured Lymphoid Tissue from Rats of Different Age

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We studied the effect of tripeptide T-38 (Lys-Glu-Asp) in the presence of cyclophosphamide on cell proliferation and apoptosis in explants of splenic lymphoid tissue from young and old rats. Peptide T-38 in a concentration of 0.05 ng/ml produced a stimulatory effect on the growth zone of the explants. Addition of 1 mg/ml cyclophosphamide to the culture medium suppressed cell proliferation, which was associated with enhanced expression of proapoptotic p53 protein. Under conditions of combined treatment with cyclophosphamide and T-38 no inhibiting effect of the cytostatics was observed. Thus, tripeptide T-38 in the presence of cytostatics produces a protective effect on cell proliferation in lymphoid tissue explants.

Key Words: *organotypic culture; lymphoid tissue; cytostatics; peptides*

The search for substances producing protective effects under conditions of impaired DNA synthesis and repair is an urgent problem of biology and medicine. Regulation of repair processes in tissues can be directed towards either stimulation of cell proliferation or its inhibition via apoptosis [5,9,11, 14]. Gene expression under these conditions are regulated by cytokines, including polypeptide growth factors, and various peptides [7,10,12]. The regulatory peptides modulating the processes of growth and development are widely distributed in living organisms and are produced by various cells and tissues as endocrine and autocrine carriers of information on local functional status of organs and tissues. However, isolation of individual peptides and evaluation of their biological activity is a labor-consuming procedure requiring analysis of activity of many hundreds of peptides. Creation of synthetic peptides enables more rapid preparation of peptides with various properties without using expensive raw materials and long-term purification procedures.

Tripeptide T-38 (Lys-Glu-Asp) was synthesized in St.Petersburg Institute of Bioregulation and Gerontology. It was previously demonstrated that addition of this peptide to the culture of the thymus from 14-16-week human fetuses increased expression of lineage-specific lymphoid and myeloid markers [2]. The targets of this peptide were $CD34^+$ hemopoietic precursor cells. T-38 increased expression of Pax1, Hoxa3, and TLR, signal differentiation factors for thymic epithelial cells (TEC); the synthesis of these factors significantly decreased in old TEC cultures. Peptide T-38 also

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increased proliferative activity of T cells cocultured with TEC.

Cyclophosphamide (CP), an inhibitor of DNA synthesis, belongs to substances with pronounced immunosuppressive properties directed towards practically all clones of immunocompetent cells (proliferating and resting) [1,3]. In light of this, it was interesting to find out whether peptide T-38 can abolish the antiproliferative effect of CP.

Organotypic tissue culture is one of the most adequate method for the screening of bioactive substances affecting the number of cells in the explant growth zone. These quantitative changes can be a criterion in primary integral evaluation of biological activity of the test substance [4,6].

Here we studied the effect of peptide T-38 in the presence of CP on the development of organotypic culture of splenic lymphoid tissue from rats of different age.

MATERIALS AND METHODS

Organotypic culturing was performed as described elsewhere [4,13]. We used 300 explants of the spleen from young (3 months) and 300 explants from old (24 months) male Wistar rats. Spleen samples isolated under sterile conditions were cut into small fragments (~1 mm^3) and placed into collagen-coated Petri dishes. The medium contained 35% Eagle medium, 35% Hanks saline, and 25% FCS and was supplemented with glucose (0.6%), insulin (0.5 U/ml) and gentamicin (100 U/ml). Evaluation of the dose dependencies showed that the effective concentrations for T-38 and CP were 0.05 ng/ml and 1 mg/ml, respectively.

The medium (3 ml) with or without the test substances in the studied concentrations were added to Petri dishes with experimental and control explants, respectively. The dishes were incubated for 3 days at 37°C and 5% CO_2 and then were examined under a phase-contrast microscope. Area index (AI) was calculated as the ratio of the explant area to the area of its central zone. The explants were visualized using a microtelevision attachment (series 10, MTN-13, Alpha-Telecom). AI was calculated using PhotoM 1.2 software. For each substance, 20-25 control and experimental explants were analyzed.

The significance of differences between the control and experimental samples was verified using Student's *t* test.

Monoclonal antibodies to p53 protein (1:75, Novocastra) were used for immunohistochemical detection of the expression of this protein. A universal kit containing biotynilated antimouse and antirabbit immunoglobulins was used. Visualization was performed with a complex of avidin with biotinylated peroxidase (ABC kit) followed by the development of horseradish peroxidase with diaminobenzidine (all reagents were from Novocastra).

Morphometry was performed using a computer-assisted microimage analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, and Videotest-Morphology 4.0 software. For each case, at least 10 fields of view were analyzed at ×400. The area of p53 expression was determined as the ratio of the area occupied by immunopositive cells to the total area of cells per field of view.

RESULTS

On day 1, we observed flattening of the explants on the collagen substrate, proliferation and migration of lymphoblasts, lymphocytes, and fibroblasts constituting the growth zone from the explant edge. After 3 days, AI of the explant increased (if the test substances produced a stimulatory effects) or decreased (in case of their inhibitory effects) compared to control values.

In experimental series I, we studied the effect of individual administration of CP or T-38 on the development of explants in organotypic culture of lymphoid tissue. For evaluation of the effect of CP on the explants from the spleen of 3- and 24-month-old rats, the drug was added to the medium to a concentration of 0.1-10 mg/ml. Starting from the concentration of 0.1 mg/ml, the preparation partially inhibited the growth zone, which significantly decreased AI by 18±3% (n=20; p<0.05) compared to the corresponding values in the control (n=22) in young rats and by 21±5% (n=25; p<0.05) compared to the corresponding values (n=23) in old rats. Increasing the CP concentration in the culture medium led to further inhibition of the explant growth. In the presence of 0.5 mg/ml CP, AI of explants decreased by 25±5% (n=21; p<0.05) compared to AI in the control (n=23). Addition of 1.0 mg/ml CP to the culture medium always significantly suppressed the development of splenic explants from young and old rats: AI decreased by 23-30% compared to AI of the corresponding control explants. Addition of T-38 peptide to the culture of lymphoid tissue increased AI of the explants by 25-38% in young animals and by 20-32% in old animals compared to the corresponding controls.

In series II, addition of 1.0 mg/ml CP to the culture of splenic tissue from 3-month-old rat pups inhibited their growth by 25±5% (n=21; p<0.05)

TABLE 1. Effect of CP and Tripeptide T-38 on AI of Splenic Explants from Rats of Different Age (*M±m*)

Substance	AI, %	Expression of p53, %
3-month-old rats		
CP	-25±5*	32±7*
T-38	26±7*	-27±5*
CP+T-38	4±1	2±1
24-month-old rats		
CP	-24±5*	28±5*
T-38	23±3*	-24±3*
CP+T-38	-7±5	5±2

Note. *$p<0.05$ compared to the control (0%).

compared to the control value (n=23). Immunohistochemical study revealed expression of proapoptotic p53 protein by 32±7% (n=22; $p<0.05$) compared to the control (Table 1). Addition of T-38 to the culture medium increased AI by 26±7% (n=20; $p<0.05$) compared to the control (n=21). Under these conditions, expression of p53 decreased by 27±5% compared to the control. However, the inhibitory effect of the cytostatics was not observed after combined treatment with CP and T-38 in effective concentrations: the growth zone of splenic explants increased by 4±1% (n=22), which is comparable with the control values of AI (n=22) and correlated with the expression of p53 protein also attaining the control level.

Addition of 1.0 mg/ml CP to the culture medium significantly decreased AI of splenic explants from 24-month-old rats by 24±5% (n=20; $p<0.05$) compared to the control (n=21); expression of p53 protein increased under these conditions. Addition of T-38 alone increased AI by 23±3% (n=20; $p<0.05$) compared to the control (n=21), while p53 expression decreased by 24±3%. Combined treatment with CP (1.0 mg/ml) and T-38 (effective concentration 0.05 mg/ml) abolished the inhibitory effect of CP on splenic explants from 24-month-old rats. The growth zone of splenic explants only insignificantly decreased and was comparable to that in the control. Expression of p53 protein did not differ from the control (Table 1).

These findings suggest that individual treatment with CP suppressed cell proliferation in lymphoid tissue explants from the spleen of young and old rats due to activation of the apoptosis processes. The toxic effect of CP is determined by its alkylating properties, *i.e.* its interaction with anions of phosphorus and carbonic acids, phenols, and with aminogroups. These radicals are widely presented in nucleic acids, enzymes, and structural proteins. Therefore, their cytostatic effect appears during G1 phase and leads to inhibition in S phase. The preparation carrying two alkylating groups forms cross-links in the DNA molecule, thus blocking DNA replication and terminating mitoses in cells, which determines the cytostatics effect of CP [3].

The results of our experiments show that tripeptide T-38 consisting of amino acids with changed side chains (acidic in aspartic and glutamic acids and basic in lysine) can abolish the inhibiting effect of CP in organotypic culture of lymphoid tissue from the spleen of young and old rats; the expression of proapoptotic protein p53 decreases under these conditions. These amino acids with low hydrophobicity and charged side chains can be simplest regulators and stimulators of physiological functions [5,8,13]. It can be hypothesized that T-38 tripeptide consisting of long amino acids can abolish the inhibitory effect of CP in the lymphoid tissue culture due to inhibition of apoptosis.

Thus, tripeptide T-38 can be recommended for further study as the preparation for elimination of immunosuppressive side effects of cytostatics, in particular in age-associated diseases.

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