Effects of Hydrophobic Amino Acids and Antibodies to Nerve Growth Factor Receptors on the Development of Splenic Tissue Culture from Young and Old Rats

N. I. Chalisova, N. A. Balikina, Yu. A. Sukhonos, and A. N. Zhekalov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 12, pp. 683-686, December, 2009
Original article submitted June 25, 2009

The effects of hydrophobic L-amino acids alone and in the presence of monoclonal antibodies to nerve growth factor receptors NGFRp75 (apoptosis inductors) were studied on organotypic culture of splenic lymphoid tissue from young (3 months) and old (24 months) rats. Nine amino acids inhibited cell proliferation in splenic explants from young rats. This was paralleled by hyperexpression of p53 proapoptotic protein. Only two amino acids stimulated apoptosis in explants from old rats. The inhibitory effects on the development of splenic explants from young and old rats were abolished in the presence of antibodies to NGFRp75. Hence, the group of hydrophobic amino acids mediates the proapoptotic effect in the lymphoid tissue of old and young rats through nerve growth factor low affinity receptors.

Key Words: *lymphoid tissue culture; amino acids; nerve growth factor receptors; aging*

Regulation of regenerative processes in tissues is effected via stimulation of cell proliferation or its inhibition by programmed cell death (apoptosis) by different proteins, including growth factors, and by bioregulatory oligopeptides. Recently, new data were obtained on the role of amino acids in the regulation of the main cell processes, proliferation and apoptosis [1,4,11,14,15]. Experiments on androgen-dependent prostatic cancer cells PC3 and DU145 showed that methionine deprivation stimulates apoptosis in PC3 cells, while tyrosine and phenylalanine deprivation stimulates this process in DU145 cells [13]. Our previous studies [2,3,5,6,9] showed that hydrophilic amino acids stimulate and hydrophobic amino acids inhibit cell proliferation in spleen organotypic culture from adult rats. Organotypic tissue culturing is one of the most adequate methods for screening the effects of bioactive substances, because the main criterion here is changed number of cells in the explant growth zone because of proliferation or apoptosis [3,4]. On the other hand, humoral and nerve factors are present in the body, but are absent in the culture. It is known that low affinity receptors to nerve growth factor (NGF) NGFRp75 belonging to the TNF family are presented on the surface of splenic lymphocytes and mononuclears [7,12]. Experiments with monoclonal antibodies and antisense oligonucleotides showed that NGFRp75 could induce apoptosis in the nervous and lymphoid tissues by regulating the expression of Bcl-2, Bcl-xl, and Fas genes [8,10].

We studied the effects of hydrophobic L-amino acids alone and in the presence of monoclonal antibodies to low affinity NGFRp75 in an organotypic culture of the spleen originating from young and old rats for evaluation of the pathways of apoptosis development.

MATERIALS AND METHODS

The study was carried out in an organotypic culture on 800 splenic explants from 3- and 24-month-old Wistar

P. Pavlov Institute of Physiology, the Russian Academy of Sciences, St. Petersburg; St. Petersburg Institute of Bioregulation and Gerontology, North Western Division of the Russian Academy of Medical Science, Russia. ***Address for correspondence:*** nata@infran.ru. N. I. Chalisova

0007-4888/09/1486-0920

rats. Fragments of the spleen (1 mm^3) processed under sterile conditions were placed in collagen-coated Petri dishes. Nutrient medium consisted from 35% Eagle medium, 35% Hanks solution, 25% fetal calf serum and contained glucose (0.6%), insulin (0.5 U/ml), and gentamicin (100 U/ml). Hydrophobic L-amino acids (aspartic acid, cysteine, tyrosine, valine, threonine, methionine, leucine, phenylalanine, tryptophan; Sigma) were added in the culture medium. Titration showed that 0.05 ng/ml was an effective concentration for all studied amino acids in lymphoid tissue culture. At lower or higher (up to 5 ng/ml) concentrations, the values of area index (AI) were statistically insignificant or at the level of the control. Mouse monoclonal antibodies to NGFRp75 (DacoCytochromation) were added in an effective concentration of 200 ng/ml (concentration blocking the stimulatory effect of 20 ng/ml NGF). Nutrient medium (3 ml) with the studied concentrations of substances was added into Petri dishes with experimental explants and the same volume of nutrient medium (3 ml) was added to dishes with control explants. Hence, explants of both groups developed in the same volume of the medium. Petri dishes were incubated in a thermostat at 37°C and 5% CO_2, delivered constantly; after 3 days, the preparations were examined under a phase contrast microscope. AI was calculated in arbitrary units as the proportion of the total explant area (together with the zone of expanding cells) to the area of the central zone. The explants were visualized with a microtelevision headpiece for microscope (series 10, MTN-13 Alpfa-Telekom). The index was calculated using PhotoM 1.2 software. A total of 20-25 experimental and 20-23 control explants were analyzed for each substance. The significance of differences in the AI of control and experimental explants was evaluated by Student's *t* test. AI was expressed in percent, control AI was taken as 100%.

Immunohistochemical detection of the expression of proapoptotic p53 protein was carried out using monoclonal antibodies to p53 (1:75; Novocastra). A universal kit of biotinylated antimouse and antirabbit immunoglobulins served as the second antibodies. The staining was visualized using avidin complex with biotinylated peroxidase (ABC-kit); horseradish peroxidase was then developed with diaminobenzidine (all reagents from Novocastra). Morphometric analysis was carried out using a system for computer analysis of microscopic images consisting of a Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, Intel Pentium 4 PC, and Videotest-Morphology 4.0 software. At least 10 visual fields at magnification 400 were analyzed in each case. The area of p53 expression was evaluated as the proportion of the area occupied by immunopositive cells to total area of cells in a visual field and expressed in percent.

RESULTS

During day 1 of culturing, the explants spread on the collagen substrate; expansion of proliferating and migrating lymphocytes and macrophages constituting the growth zone from the explant edge was observed. If the development of the growth zone was inhibited, AI decreased in comparison with the control.

Addition of each of the amino acids to the culture medium with young rat explants significantly inhibited the development of the explant growth zone (Table 1). AI decreased by 29±3% (n=21; p<0.05) under the effect of 0.05 ng/ml tyrosine in comparison with the control (n=24). Addition of 0.05 ng/ml valine, threonine, or methionine resulted in a similar statistically significant reduction of the growth zone area, which was seen from AI decrease by 35±5% (n=23; p<0.05), 35±7% (n=25; p<0.05), and 35±3% (n=20; p<0.05), respectively, in comparison with the control (n=22, n=21, and n=20, respectively). Aspartic acid reduced AI by 39±9% (n=20; p<0.05), cysteine by 36±7% (n=23; p<0.05) in comparison with the control (n=21 and n=24, respectively). Only two of the studied amino acids inhibited cell proliferation in explants from old 24-month-old rats. Addition of threonine led to reduction of AI by 26±5% (n=25; p<0.05) and tryptophan reduced it by 32±7% (n=22; p<0.05) in comparison with the control (n=24 and n=23, respectively). Study of the expression of proapoptotic protein p53 showed a statistically significant increase in the area of p53 expression under the effect of all amino acids except cysteine, tyrosine, and valine. Aspartic acid, threonine, methionine, leucine, phenylalanine, and tryptophan demonstrated a clear-cut negative correlation between the decrease of AI value and increase of the area of p53 expression by 51-80% (Table 1). The expression of p53 increased in explants from old (24-month old) rats by 59-68% under the effects of proliferation-inhibiting threonine and tryptophan.

In the next series of experiments we studied the impact of NGFRp75 blockade for the amino acid effects. Antibodies to NGFRp75 added to the culture medium in an effective concentration inhibited the development of splenic explant growth zone by 30±3% (n=21; p<0.05) compared to the control (n=23). Combined treatment with amino acids and antibodies to NGFRp75 led to different results. Addition of each of the hydrophobic acids together with antibodies to NGFRp75 to culture medium canceled the inhibitory effects of amino acids. The explant growth zone in these cultures was comparable to the growth zone in the control, the AI values not differing from the control in explants from young and old animals. Immunohistochemical study of the expression of p53 (proapoptotic protein) showed a statistically

TABLE 1. Effects of Amino Acids (AA) and Their Combinations with Antibodies to NGFRp75 on AI and Areas of p53 Expression in Rat Spleen Explants (*M*±*m*)

Amino acid	AI, %	Expression of p53, %	AA+NGFRp75	
			AI, %	p53 expression, %
3-month-old rats				
Aspartic acid	-39±9*	51±11*	-12±3	-44±7*
Cysteine	-36±7*	10±5	4±1	-41±13*
Tyrosine	-29±3*	4±2	-14±3	-40±11*
Valine	-35±5*	3±1	5±1	-45±15*
Threonine	-35±7*	78±14*	12±5	-58±10*
Methionine	-35±3*	80±13*	10±6	-70±9*
Leucine	-36±9*	77±11*	9±3	-62±13*
Phenylalanine	-32±7*	56±12*	6±1	-65±14*
Tryptophan	-30±5*	60±15*	5±2	-55±12*
24-month-old rats				
Threonine	-28±5*	68±12*	7±3	-50±11*
Tryptophan	-32±7*	59±14*	5±1	-52±15*

Note. *$p<0.05$ compared to the control (100%).

significant reduction of p53 expression zone after combined treatment by amino acids and antibodies to NGFRp75. A 40-45% decrease in p53 expression area was observed for medium hydrophobic amino acids (aspartic acid, cysteine, tyrosine, valine). Combinations of highly hydrophobic amino acids (threonine, methionine, leucine, phenylalanine, tryptophan) with antibodies to NGFRp75 significantly inhibited p53 expression. Phenylalanine in combination with antibodies to NGFRp75 reduced AI by 65±14% (n=20; $p<0.05$) in comparison with the control (n=21), methionine in the same combination caused a 70±9% reduction (n=24; $p<0.05$) in comparison with the control (n=23). The expression of p53 under the effect of highly hydrophobic amino acids in the presence of antibodies to NGFRp75 was by 1.5 times higher than under the effects of amino acids with medium hydrophobia. The decrease in explant AI under the effect of antibodies to NGFRp75 alone can be attributed to attenuation of the effects of growth factors present in fetal serum due to blockade of NGF receptors. Potentiation of the inhibitory effects on the expression of proapoptotic p53 in combined treatment by amino acids and antibodies to NGFRp75 suggests that the approaches to apoptosis regulation are common for highly hydrophobic amino acids and antibodies to NGFRp75, presumably due to regulation of Bcl-2, Bcl-x genes expression [11,12].

In our experiments, the effective concentration for amino acids was 0.05 ng/ml (10^{-12} M), which can be attributed to the effect of ultralow doses, focusing the attention of scientists at present. The effects of amino acids were realized in the presence of amino acids of the nutrient medium, which confirms the concept according to which it is not the absolute concentration of bioactive substances that is important, but slight shifts in this concentration [3,14].

Our findings indicate that, despite the decrease in the number of active amino acids in the explants from old rats, the mechanisms of modulating effects of amino acids on the cell processes are similar in lymphoid tissue of young and old animals. The results indicate that the regulatory effects of highly hydrophobic amino acids depend on the presence of growth factor receptors. These data should be taken into consideration when creating new regulatory peptides, which can modulate cell proliferation and apoptosis in tissues. These data form the basis for targeted creation of bioregulatory peptides stimulating the regeneratory processes in damaged tissues and apoptosis in tumor diseases.

REFERENCES

1. G. A. Belokrylov, O. N. Derevnina, O. Ya. Popova, *et al.*, *Byull. Eksp. Biol. Med.*, **121**, No. 5, 509-512 (1996).

2. V. Kh. Khavinson and V. V. Malinin, *Med. Akad. Zh.*, **8**, No. 1, 145-151 (2008).
3. N. I. Chalisova and A. V. Komashnya, *Bioorg. Khim.*, **32**, No. 3, 293-299 (2006).
4. N. I. Chalisova, V. V. Lesnyak, G. A. Ryzhak, and E. V. Oganezova, *Byull. Eksp. Biol. Med.*, **145**, No. 6, 605-608 (2008).
5. N. I. Chalisova, V. A. Penniyainen, and A. D. Nozdrachev, *Dokl. Akad. Nauk*, **393**, No. 2, 1-5 (2003).
6. N. I. Chalisova, V. A. Penniyainen, N. V. Kharitonova, and A. D. Nozdrachev, *Ibid.*, **380**, No. 3, 418-421 (2001).
7. G. L. Barrett, A. Georgiou, K. Reid, *et al.*, *Neuroscience*, **85**, No. 4, 1321-1328 (1998).
8. M. Canete, A. Juarranz, P. Lopez-Nieva, *et al.*, *Acta Histochem.*, **103**, No. 2, 117-126 (2001).
9. N. I. Chalisova and A. N. Zakutzkii, *Cell Biol. Int.*, **32**, No. 12, 1574-1577 (2008).
10. S. S. Cheema, G. L. Barrett, and P. F. Bartlett, *J. Neurosci. Res.*, **46**, No. 2, 239-245 (1996).
11. A. M. Conti, S. Brimijoin, L. J. Miller, and A. J. Windebank, *Neurobiol. Dis.*, **15**, No. 1, 106-114 (2004).
12. C. Culmsee, N. Gerling, M. Lehmann, *et al.*, *Neuroscience*, **115**, No. 4, 1089-1094 (2002).
13. M. Fratelli, V. Gagliardini, G. Galli, *et al.*, *Blood*, **85**, No. 12, 3532-3537 (1995).
14. L. S. Jefferson and S. R. Kimball, *J. Nutr.*, **131**, No. 9, Suppl., 2460S-2466S (2001).
15. K. S. Lowry and S. S. Sheema, *In Vitro Cell Dev. Biol. Anim.*, **36**, No. 8, 520-526 (2000).