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THE ROLE OF AUTOPHAGY PROCESSES IN MAINTAINING VITAL ACTIVITY OF TUMOUR CELLS UNDER CONDITIONS OF STRESS ON THE PATTERN OF MELANOMA CELL LINE

Abstract. *Due to autophagy cells are able to supply the lack of nutrients and energy under stressful conditions and return to normal vital activity. Autophagy does not only promote enhancing of vital activity of cells but their death as well. A transition moment between accumulation of the reserve abilities of cells with the purpose to preserve vital functioning and the onset of autophagic type of cellular death still remains unknown. The knowledge of autophagy processes is especially important to understand pathogenesis of oncological diseases. The study was conducted on 793 pattern of melanoma cell line cultivated under stressful conditions simulated by changing culture environment into decreased level of nutrients in different terms of time. Autophagy processes were investigated in our study with addition of rapamycin (sirolimus) and chloroquine, and the role of EGFR in maintaining vital activity of cells. A therapeutic effect of target EGFR on 793 modeled cell line of melanoma was proved.*
Key words: *autophagy, apoptosis, EGFR, rapamycin (sirolimus), chloroquine, melanoma.*

Introduction. Autophagy is a process when internal components of the cell are transmitted inside of its lysosomes or vacuoles and experience degradation in them [1]. The role of autophagy in maintaining vital activity of cells and activation of their death under stressful conditions have been unknown for a long time, and only in 2016 the Japanese researcher Yoshinori Ohsumi was awarded the Nobel Prize in physiology and medicine due to discovery of the main regularities of this process.

The main stimuli promoting occurrence of autophagy processes in cells can be the following: lack of nutrients, availability of damaged organelles, partially denatured proteins or their aggregates in the cytoplasm. Autophagy can be also induced by oxidative or toxic stress. In general, due to autophagy cells are able to supply the lack of nutrients and energy under stressful conditions and return to normal vital functioning [2].

There are three types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy and chaperone-mediated autophagy are activated only under stressful conditions. Therefore, they attract the most attention for further detailed investigations. In case of macroautophagy the cytoplasm containing any organelles is surrounded by the membrane compartment similar to the cistern of the endoplasmic network. As a result, this part is separated from the rest of the cytoplasm by two membranes. Such structures with double layer membranes are called autophagosomes. Autophagosomes are combined with lysosomes forming autophagolysosomes, in which organelles and the rest of the content of autophagosomes are digested [3].

Chaperone-mediated autophagy is characterized by a directed transport of partially denatured proteins from the cytoplasm through the lysosome membrane into its cavity where they are digested. This process occurs with participation of cytoplasmic proteins-chaperones of hsc-70 family, auxiliary proteins and LAMP-2,

serving as a membranous receptor of the chaperones-protein complex awaiting their transport into the lysosome.

Autophagy does not only promote enhancing of vital activity of cells but their death as well. In case of autophagic type of cellular death all the organelles in the cell are digested, and remaining cellular debris is completely absorbed by macrophages [4]. A transition moment between accumulation of the reserve abilities of cells with the purpose to preserve vital functioning and the onset of autophagic type of cellular death still remains unknown.

The knowledge of autophagy processes is especially important to understand pathogenesis of oncological diseases, as from one side it is the method of survival of cells, and from another side the method of activation of cellular death, which in a number of cases give a perspective of sensitization of tumour cells [5].

With this purpose many scientists deal with investigations of activators and inhibitors of autophagy. The mechanism of autophagy action (rapamycin, tamoxifen, perifosine, erlotinib, etc.) is mainly based on inhibition of mTOR-signal way or increased expression of *Atg* autophagy genes, while inhibitors (bafilomycin A, chloroquine) prevent fusion of autophagosomes with lysosome [6, 7]. EGFR blocking is used in practical medicine with the aim to treat and prevent relapses of oncological diseases, although the signs of autophagy processes in the course of treatment of oncological patients still remain unknown [8].

Objective. To study the role of autophagy in maintaining vital activity of cells under stressful conditions and with the use of antibodies to EGF on the pattern of melanoma cell line.

Materials and methods. The experiment was conducted on 793 melanoma cell line obtained from ATCC (Great Britain). The cells were kept at the temperature -80˚C. The cells were reproduced on the water bath at the temperature +37˚C followed by centrifugation during 5 minutes at 1000 × g (Heraeus Biofuge). The received supernatant was isolated and added to 1 ml of cellular suspension in 4 ml of culture medium RPMI 1640 in the flask for cultivation (JET BIOFIL TCF-012-050). The work with cells was performed in safety cabinet (Steril-VBH). The cells were calculated by means of the microscope Leica (within the norm no less than 3×10^5 cells / ml). Unfrozen cells were incubated in CO_2 incubator at the temperature of + 37 ˚C (5 % CO_2, Heraeus).

72 hours later RPMI 1640 was changed for isolation of cryopreservative. 7 days after changing the culture medium the cells were distributed into 4 groups. At the beginning of the experiment every group of 793 melanoma cell line grew in the culture medium RPMI 1640 with addition of 10 % FBS (fetal bovine serum), 2 mM L-glutamine and 1% Pen-Strep (penicillin-streptocid) (Sigma-Aldrich, USA). In the first group of cell stressful conditions were simulated on the 4, 8, 12 and 24 hour, reproducing starvation conditions by means of changing culture medium into the medium with low content of nutrients (RPMI 1640 with addition of 0,1 % FBS, 2 mM L-glutamine and 1 % Pen-Strep). The second group of cells was cultivated in saturated culture medium RPMI 1640 with addition of 10 % FBS, 2 mM L-glutamine, 1 % Pen-Strep, which was changed on the 4^{th} hour with addition of 10 nM rapamycin, on the 8^{th} hour – with addition of 50 nM rapanycin, on the 12^{th} hour – with addition of 75 nM rapamycin, and on the 24^{th} hour – with addition of 100 nM rapamycin (Sigma-Aldrich, USA). In the third group of cells stressful conditions were simulated on 4, 8, 12 and 24 hour, reproducing starvation conditions by means of changing culture medium into the medium with reduced content of nutrients and 10 nM chloroquine (Sigma-Aldrich, USA). In the fourth group of cells stressful conditions were simulated on 4, 8, 12 and 24 hour, reproducing starvation conditions by means of changing culture medium into the medium with reduced content of nutrients and antibodies to EGF in the concentration 0,2 μg/mL (Sigma-Aldrich, USA). All the groups of cells were incubated in CO_2 incubator at the temperature + 37 ˚C (5 % CO_2, Heraeus).

To assess the vital functioning of the examined groups of cells colorimetric test was made with tetrazolium staining agent 3-(4,5-dimethylthiazole-2-il)-2,5-diphenyl-tetrazolium bromide (MTT-test) according to the standard procedure (Sigma-Aldrich, Saint Louis, MO, USA) [9]. Colour intensity (optic density) was measured at 530 nm by means of microplanchette (Infinite F50, TECAN). The experiment was conducted

three times.

The results were statistically processed by means of dispersive and correlation analysis using the standard functions of the package MS Excell 2010 (Microsoft Inc., USA). Criterion values and the main calculations were made by means of the specialized software ANOVA (Stat View 4.0 software, Abacus Concepts, Berkeley, CA, USA). As the criterion of difference credibility of the indices was significance level P < 0,05.

Results and discussion. Proliferation of cells of 793 melanoma cell line was determined by means of MTT-test by the colour intensity level. At the beginning of the experiment the vital activity of cells was similar in every group of 793 melanoma cell line and was accepted as 100 % (Table 1, Figure 1).

In the control group under stressful conditions reproduced by means of changing culture medium with decreased level of nutrients in different period of time, the vital activity of cells decreases gradually, although at the end of the experiment it was higher as compared to other groups (P < 0,05). Thus, on the 4th hour of starvation the vital activity of cells decreased by 10 %, on the 8th hour – by 20 %, on the 12th hour – 25 %, and on the 24th hour – by 28 % in comparison with the beginning of the experiment (P < 0,05). Although cellular proliferation rate in the control group began to decrease on the 12th hour of starvation, and it was the lowest on the 24th hour. At this term the vital activity of cells became only 3% lower in comparis on with the same value on the 12th hour. The highest decrease of cellular proliferation rate

Table 1.

Assessment of the vital activity of melanoma cell line against the ground of induction and inhibition of autophagy processes under stressful conditions in 4, 8, 12 and 24 hours since the beginning of cultivation (M±m, %).

	Groups	Beginning of the experiment	Stressful conditions (starvation)			
			4 hours	8 hours	12 hours	24 hours
1.	Control	100±0,23	90,99±0,27	79,78±0,11	75,97±0,16	72,87±0,21
2.	Cells with rapamycin	100±0,33 (0 nM)	66±0,19 (10 nM)	62±0,17 (50 nM)	56±0,1 (75 nM)	54±0,22 (100nM)
3.	Cells with chloroquine	100±0,12	64,29±0,22	57,89±0,25	40,81±0,32	54±0,21
4.	Cells with anti-EGF	94,47±0,15	81,12±0,14	78,38±0,23	75,96±0,27	64,88±0,31

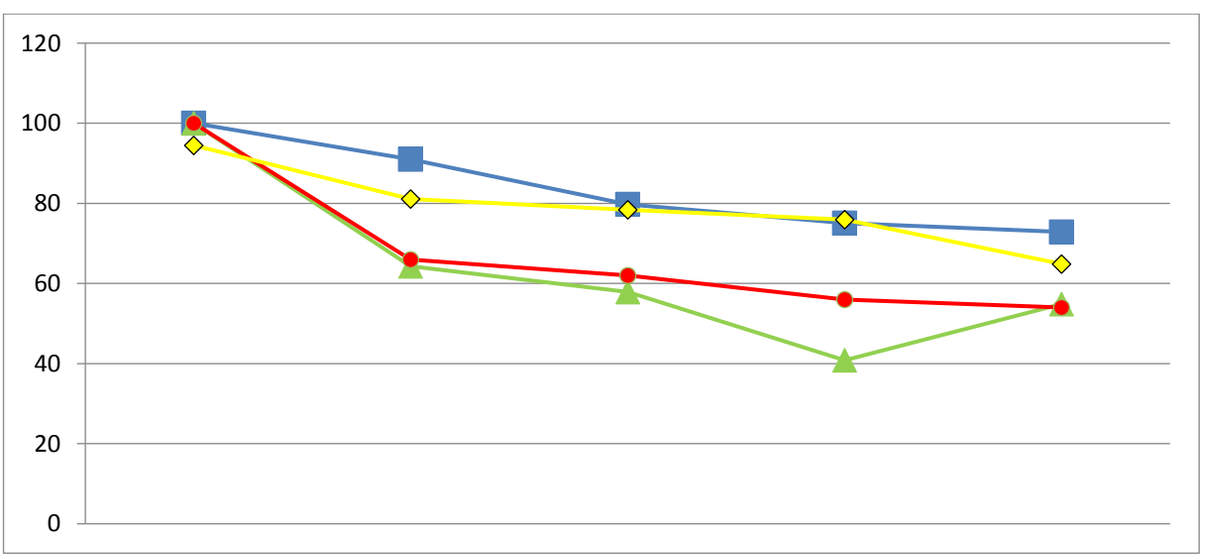

Fig.1. Vital activity of melanoma cell line against the ground of activation and inhibition of autophagy processes under conditions of stress in 4, 8, 12 and 24 hours since the beginning of cultivation (P < 0, 05). Signs: ■ control; ● cells with rapamycin (10, 50, 75, 100 nM); ▲ cells with chloroquine; ◆ cells with antibodies anti-EGF

occurred in the period from 4 to 8 hour of starvation, while the vital activity of cells became 10% lower. Therefore, against the ground of starvation beginning with the 12th hour cells begin to adapt to stressful conditions. Dephosphorilation of mTOR-signal way probably occurs exactly at this period inducing increased vital activity of cells by means of autophagy activation. Although, at the beginning of occurring stressful conditions intensive destruction of cells is observed which is indicative of activation of autophagic death of cells or apoptosis, preceding the increase of general vital activity of cells. Nevertheless, molecular mechanisms of this transmission remain unknown and require further investigations.

Against the ground of additional administration of rapamycin as an autophagy activator in different concentrations (10, 50, 75 and 100 nM) to culture medium without simulating stressful conditions the vital activity of cells reduced considerably as well as compared to the experiment. Thus, addition of rapamycin in the concentration of 10 nM promoted decreased cell proliferation as much as 34%, in the concentration of 50 nM –38 % less, in the concentration of 75 nM – 44 % less, and in the concentration of 100 nM –46 % less as compared to the beginning of the experiment ($P < 0,05$). Therefore, the lowest vital activity of cells was found with the concentration of rapamycin in the dose of 10 nM, where cell proliferation was in 1,5 times less than in the group of cells without administration of rapamycin ($P < 0,05$). The highest decrease of the vital activity of cells was found with the concentration of rapamycin of 100 nM, where cell proliferation was by 1,9 times lower than in the group of cells without rapamycin ($P < 0,05$). Therefore, a conclusion can be drawn that the more intensive dephosphorilation of mTOR-signal way occurs, the more intensive activation of autophagic type of cell death is. Although in case the block of this signal way is not considerable, the rate of proliferative properties decreases less. Thus, the degree of activation of autophagy depends on the intensity and time of block of mTOR-signal way. Probably at the beginning of starvation death of cells occurs, since dephosphorilation of mTOR-signal way is not sufficient and autophagy processes fail to activate reserve abilities of cells to maintain their vital activity. In case dephosphorilation of mTOR-signal way is appropriate, autophagy is activated promoting maintenance of the vital activity of cells. Although, the more intensive dephosphorilation of mTOR-signal way occurs, the more probable autophagic death of cells is. Probably at the beginning of stressful conditions the processes of cell apoptosis are activated followed by autophagic inhibition directed to the increase of the vital activity of cells. And as far as all possible resources are exhausted autophagic death of cells occurs. Therefore, activation of apoptosis with the purpose to treat oncological diseases can promote increased cell resistance, and then this suggestion requires more detailed investigation.

When chloroquine, as autophagy inhibitor, is added with simulation of stressful conditions on the 4, 8, 12 and 24th hour a considerable decrease of the vital activity of cells occurs as compared to the cells from the control group cultivated under the similar stressful conditions ($P < 0,05$). On the 12th hour of starvation in the group of cells with chloroquine the lowest index of cell proliferation is found (40,81 %), which was in 1,86 times less than in the control group with simulation of stressful conditions at the same time ($P < 0,05$). Thus, undoubtedly autophagy increases the vital activity of cells, and the highest activity of this process occurs on the 12th hour of starvation. It is also evidenced by decreased intensity of vital activity loss exactly on the 12th hour of starvation in the control group.

The use of antibodies to EGF, which in the norm should activate EGFR and, therefore, stimulate phosphorilation of mTOR-signal way causing intensive growth of cells, and considerable inhibition effect on cell proliferation in comparison with the control group was not provoked ($P < 0,05$). In case of dephosphorilation of mTOR-signal way by means of antibodies to EGF inconsiderable decrease of the vital activity of cells was found on the 4th hour in 1,12 times in comparison with the control group under the similar stressful conditions ($P < 0,05$). Thus, when EGFR stimulation is absent proliferation of melanoma cells decreases, and stressful conditions on the 4th hour do not cause activation of autophagy. Although on the 8th and 12th hour of

cell starvation there was no difference in cell proliferation in both groups found. On the 24th hour of simulation of stressful conditions the vital activity of cells was lower than in the control group by 1,12 times (P < 0,05). Thus, with EGFR block against the ground of stress on the 8th hour other signal ways are involved which are directed to autophagy activation and maintenance of the vital activity of cells. Review of literary sources gives the ground to predict that a central role in this process belongs to JNK-signal way (c-jun N-terminal kinase). Different stress factors are able to activate JNK by means of conformation disorders of sensitive phosphatases which in the norm inhibit this signal way and proteins activating it. In its turn, JNK activation promotes phosphorylation of Beclin 1 protein which is a trigger of autophagy. JNK can work as promoters of cell apoptosis. And JNK role in activation of apoptosis depends on the type of cells and kind of stimulus. The influence of JNK activation on apoptosis was suggested to depend on the activity of other signal ways, for example, ERK or NFkB-mediated, which enables to suggest that JNK activation facilitate but not initiate apoptosis process [10, 11, 12]. Although, decrease of the vital activity of cells with the use of antibodies to EGF on the 24th hour of starvation of cells gives the evidence of therapeutic effect of the target EGFR therapy for the patients with melanoma, and transmission of JNK from autophagy to the maintenance of apoptosis in case of more prolonged effect of stressful conditions.

Conclusions: Increase of the vital activity of cells of 793 melanoma cell line under stressful conditions occurs on the 12th hour of starvation.

1. With the concentration of 10 nM of rapamycin proliferation of 793melanoma cell line decreases in 1,5 times as compared to the cells without administration of rapamycin (P < 0,05), which is the lowest index of decreased cell proliferation when rapamycin is administered. With 100 nM of rapamycin proliferation of 793 melanoma cell lien decreases in 1,9 times in comparison with cells without administration of rapamycin (P < 0,05), which is the highest index of decreased cell proliferation when rapamycin is used.

2. On the 12th hour of starvation in the group of 793 melanoma cell line with the concentration of 10 nM of chloroquine the highest decrease of cell proliferation occurs (40,81 % less), which is in 1,86 times less than in the group of cells with simulation of stressful conditions at the same time (P < 0,05). The highest inhibition of autophagy processes on the pattern of 793 melanoma cell line occurs on the 12th hour of simulation of stressful conditions.

3. On the 24th hour of simulation of stressful conditions with the use of antibodies to EGF the vital activity of 793 melanoma cell line is lower than that in the control group in 1,12 times (P < 0,05). Therapeutic effect of target EGFR therapy of 793 melanoma cell line under stressful conditions is found on the 24th hour.

Perspectives of further studies. To investigate the expression of autophagy markers in comparison with expression of proteins of EGFR – signal way under stressful conditions with the purpose to find objects for target therapy of melanoma directed to inhibition of autophagy processes is rather perspective. Examination of trigger mechanisms of autophagy activation and transition to autophagic cell death could supplement the knowledge concerning pathogenesis of oncological diseases and promote the development of new approaches of personalized medicine to the treatment of oncological patients.

References:

1. Vellai T, Bicsa´k B, To´th, ML, et al. Regulation of cell growth by autophagy. Autophagy 2008; 4: 507-9.

2. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell 2011; 147: 728-41

3. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res 2014; 24: 24-41.

4. Qian HR, Shi ZQ, Zhu HP et al. Interplay between apoptosis and autophagy in colorectal cancer. Oncotarget. 2017; 8(37): 62759-68.

5. Amaravadi RK, Lippincott-Schwartz J, Yin XM et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin. Cancer Res 2011; 17 : 654-66.

6. Rubinsztein DC, Shpilka T, Elazar Z. Mechanisms of autophagosome biogenesis. Curr. Biol 2012; 22 : 29-34.

7. Zhu J, Zheng Y, Zhang H et al. Low

concentration of chloroquine enhanced efficacy of cisplatin in the treatment of human ovarian cancer dependent on autophagy. Am J Transl Res.2017; 9(9): 4046-58.

8. Tan X, Thapa N, Sun Y, Anderson R. A Kinase Independent Role for EGF Receptor in Autophagy Initiation. Cell 2015; 160 (0): 145-60.

9. Berridge MV, Herst PM , Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnology Annual Review 2005; 11: 127-52.

10. Henson E, Chen Y, Gibson S. EGFR family members' regulation of autophagy is at a crossroads of cell survival and death in cancer. Cancers 2017; 9: 27.

11. Wei Y, Zou Z, Becker N et al. EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. Cell 2013; 154: 1269-84.

12. Yu P, Zhang C, Gao CY, Ma T et al. Anti-proliferation of triple-negative breast cancer cells with physagulide P: ROS/JNK signalingpathway induces apoptosis and autophagic cell death. Oncotarget 2017; 8(38): 64032-49.