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The Influence of Nimotuzumab in Combination with EGF on the Cell Cycle and Apoptotic Level of Tumor Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author LVG designed the study and wrote the first draft of the manuscript. Author OID wrote the protocol. Authors VVN and OVS managed the analyses of the study. Author DVS performed the statistical analysis. Author TVN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of study was to show the combined influence of nimotuzumab (NTM) and EGF on the proliferation, apoptosis by breast cancer cells line MCF-7.
Methodology: Cell line was cultivated in DMEM under standart conditions. Nimotuzumab and EGF was added to the cells which reached 70-80% monolayer. Flow cytometry assay was conducted to determine cell division in the phases of cell cycle and apoptosis. Cell viability was assessed by MTT test and routine calculation cells used Gor'yaev chamber test with trypan blue.
Results: Obtained results show that the efficiency of the combined effects on tumor cells of breast cancer MCF-7 antibodies to epidermal growth factor receptor – nimotuzumab, in combination with epidermal growth factor enhances the cytotoxic/cytostatic effect of NTM. According to the data, NTM appeared a cytostatic, cytotoxic and proapoptotic effects. Thus, after NTM addition to cultured

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cells, relative quantity of cells in phases G0 / G1 were increased in 1.2 times (P<0.05), compared to control, the percentage of apoptotic cells was 4-fold increased (P<0.01), and dead cells - almost 2-fold (P<0.05). Previous incubation of cells with EGF (5 nM) during 2 hours before adding NTM was characterized by an increase in the relative content of cells in G0 / G1 phase to 78,3±2,2%, against 73,4±1,4% NTM impact, 58,8±2,1% in the control and 38,9±0,9% for EGF influence.
There was also a redistribution of concentration of apoptotic and dead cells: reducing the percentage of apoptotic compared to the influence of the mono NTM and increase dead cells, respectively.

Keywords: Tumour cells; breast cancer; epidermal growth factor; nimotuzumab.

1. INTRODUCTION

Strategies for combination of therapeutic anticancer action today include different effects on molecular and cellular targets, that are involved in the progression of tumors and resistant to proapoptotic signals of endogenous and exogenous signals [1-2]. The most studied mechanism of inhibition of apoptosis as mechanism of malignant transformation is increase autocrine and paracrine expression of growth factors and / or their receptors on the cells of the tumor clone. Most peptide growth factors show mitogen properties. They can act as autocrine and / or paracrine metabolic loops to determining the degree of malignancy of tumors [3-5]. Receptors with tyrosine kinase activity, which specific mitogen induction is mainly directed by autocrine mechanism of tumor growth, is one of the main targets of selective anticancer therapy [2,6]. EGFR - tyrosine kinase receptor of epidermal growth factor, the functional role of which is related to adhesion, proliferation and migration of cells [6-8]. Increased expression of EGFR is observed in 30% of human carcinomas and correlated with the level of tumor invasion and metastasis [7,8]. In particular, consider that ErbB1 plays an important role in the development of lung cancer and colon. ErbB2 (or HER2) serves as a potent oncogene that involved in the pathogenesis of breast cancer [9]. Therefore, inhibition of expression of EGFR family receptors is one of the widely used techniques antitumor effects for today. Example of targeted therapy, which is a target ligand - EGFR system, is using Herceptin - humanized monoclonal antibody to the second type of EGF receptor (ErbBII/Her 2 neu) [9-12]. Targeted anticancer therapy indicated direction includes blocking receptors by specific monoclonal antibodies, inhibition of tyrosine kinases of this receptors, using conjugates of "ligand-toxin" with cytotoxic activity, non-specific blocking receptors by antagonists, using of anti sensory oligonucleotides which directed to receptors or ligands expression regulation [8-10]. One of the perspective directions of solving the problem of inhibiting proliferation of malignantly transformed cells is the development of conjugates of cytotoxic drugs with polypeptides vectors [13]. Selectivity of transport in this case is achieved by the ability of the ligand (vector) to the high affinity binding to specific receptors which expressed only / mostly on target cells. However, creating dosage forms of drugs which based on these conjugates were requires additional technological development, the study of the pharmacodynamics, pharmacokinetics, and more. But, a significant problem in anticancer therapy are tumor cells that are in G0-phase of the cell cycle, since bioavailability to them chemotherapy and humanized antibodies is lower than the cells of proliferative pool (S + G2 / M). Therefore, developing additional approaches to the use of compounds that can synchronize tumor cells in the synthetic phase (S), which sensitivity is high to anticancer drugs (that are intercalating DNA); to anti-tubulin drugs (G2-M transition); increase sensitivity to p53-induced apoptosis and others. Therefore, in this study as a stimulator of exogenous DNA synthesis of MCF-7 cells (breast adenocarcinoma) with high EGFR expression, ligand of EGFR - EGF was used. Its effect on cells was assessed in combination with NTM - anticancer drug, which possessed the effect to inhibit of kinase-dependent EGFR phosphorylation, that lead to arrest the cell cycle in G1-phase. Thus, the purpose of study was to determine the cytotoxic / cytostatic, proapoptotic NTM influence in combination with EGF relative to MCF-7 cells.

2. MATERIALS AND METHODS

MCF-7 cells were incubated in the culture media DMEM (Sigma, USA) with 10% FBS (Sigma, USA), 2 mM L-glutamine, and 40 µg/ml gentamicin under normal conditions (5% CO_2, 100% humidity, 37ºC). Test compound were added to the cells which reached 70-80%

monolayer. Cytotoxic / cytostatic, antiproliferative effect and cell viability under influence of NTM in concentration 0,15-15 mg/ml was determined in the MTT-test [14,15] after inclusion in living cells soluble in physiological buffers dye-3-(4,5-dimethyl thiazol-2-il)-2,5-diphenyl-2-tetrazolium bromide and transformation mitochondrial dehydrogenases in insoluble purple crystals of formazan. After the cell dissolution in dimethylsulfooxide, the concentration of living cells was determined by the intensity of the color in the colorimetric analysis at a wavelength of 570 nm. Value of live and dead cells by the inclusion of recent trypan blue dye was determined by routine counting in the Gor'yaev chamber. The distribution of tumor cells in different phases of the cell cycle and the relative number of apoptotic cells was assessed by flow cytometry [16] under influence of NTM (1,5 mg/ml), EGF (5 nM); for determining the combined impact, EGF contributed to the incubation medium at a concentration of 5 nM for 2 hour before making NTM. For this purpose the samples were stained with PI, which selectively joins with intercalating places in DNA. Cytofluorymetry was carried out on the instrument FACS Calibur (Becton Dickinson, United States). Special mathematical program Mod Fit LT 2.0 (BDIS, United States) for Macintosh computers was used for acquisition and data analysis. Narrowband filter 585/42 nm was used in order to measure the fluorescence of PI. Substrate dependence and cell morphology was evaluated after staining by Crystal Violet as described previously [17]. Investigated parameters were determined after 48 hours of the cells incubation with agents. MCF-7 cells incubated under the same conditions without agents were used as control. Cytotoxic effect was determined by the percentage of dead cells, cytostatic - relative content of cells in G0-phase and concentration of living cells relative to control. Substrate dependence determined by the concentration of attached cells to the substrate. Statistical analysis was carried out by Student'st-criterion. Values of $P < 0.05$ were regarded as statistically significant.

3. RESULTS AND DISCUSSION

There was detected that highest cytotoxic / cytostatic effect was in concentration of 1,5 mg/ml NTM (data is not shown) by MTT-test. Based on this we verified the influence of NTM in IC50/10 concentration ie in 1,5 mg/ml concentration.

According to these data, a cytotoxic / cytostatic effect of NTM in combination with EGF relative to MCF-7 cells was 56% ($P<0.05$) comparatively to the control, while NTM adding led to a reduction of living cells, and equations to the control by 45% ($P <0.05$), (Fig. 1).

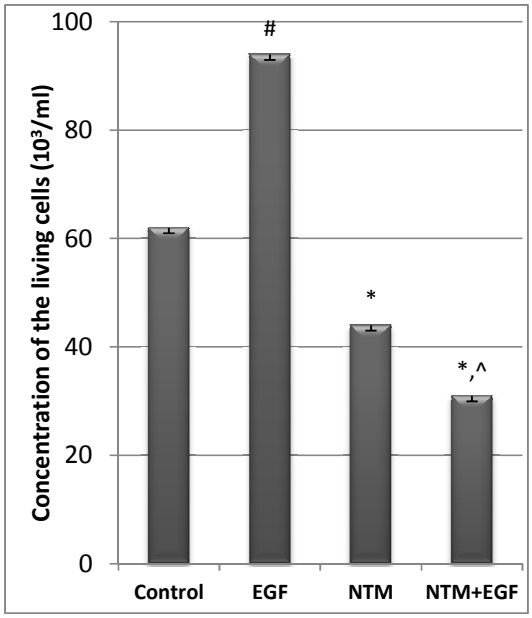

Fig. 1. The concentration of living MCF-7 cells under the influence of nimotuzumab (NTM) and NTM in combination with EGF. The term incubation of cells with agents and in control - 48 hours

** - P <0.05, compared with control, ^ - P <0.05, compared with NTM; # - compared to EGF*

As seen from the above data, the concentration of living cells determined by routine counting and MTT-test, the impact of NTM decreased in 1.2 times ($P<0.05$) *vs* control, whereas in the joint influence of NTM and EGF, the characteristic was lower by half ($P <0.05$) *vs* the control and 1.6-fold ($P <0.05$) against NTM, and almost three times - compared with the effect of EGF.

In determining the phases of cell division of the cell cycle, 58,8±2,1% of which were in the G0 / G1 phase in control (Table 1), there was shown that test agents had different impact on this index.

According to the data, NTM had a cytostatic, cytotoxic and proapoptotic effects (Tables 1 and 2). So, when we added to cultured cells NTM, the relative number of cells in phases G_0 / G_1 increased at 1.2 times ($P <0.05$), compared to the control, the percentage of apoptotic cells

increased 4-fold (P<0.01), and dead cells - almost 2-fold (P<0.05). Previous incubation of cells with EGF (5 nM) by 2 hours before adding NTM was characterized by an increase in the relative content of cells in G_0 / G_1 phase to 78,3±2,2%, against 73,4±1,4% impact on NTM, 58,8±2,1% in the control and 38,9±0,9% for the influence of EGF. There was also a redistribution of concentration of apoptotic and dead cells: reducing the percentage of apoptotic compared to the influence of the only NTM and increase dead cells, respectively.

In determining the morphological characteristics and formation of adhesion locuses for the actions of all studied test–samples of cells after washing by physiological buffers painting by Crystal Violet as described [17]. As can be seen from the picture (Fig. 2), the impact of mono NTN and in combination with EGF was observed clusters of cells with units of unique multi cellular spheroids in some areas.

Consequently, studies with using cultured tumor MCF-7 cells with high EGFR expression showed that cytotoxic / cytostatic effect of joint action of specific EGFR ligand in combination with a targeted drug aimed at increasing EGFR. Defining mechanisms of combined effects of EGF with NTM requires further research.

Table 1. Distribution of MCF-7 cells by cell cycle phase under the influence of NTM and EGF applied separately and in combination

Test samples	G_0/G_1, %	S, %	G_2/M, %
Control	58,8±2,1	32,6±1,3	8,4±1,6
EGF	38,9±0,9*	46,9±1,4*	14,0±1,9
NTM (1,5 mg/ml)	73,4±1,4*	25,3±3,1	3,0±0,3*
NTM (1,5 mg/ml) + EGF (5 nM)	78,3±2,2*,^	20,4±1,3*	3,9±1,3*

** - P <0.05, compared with control, ^ - P <0.05, compared with NTM*

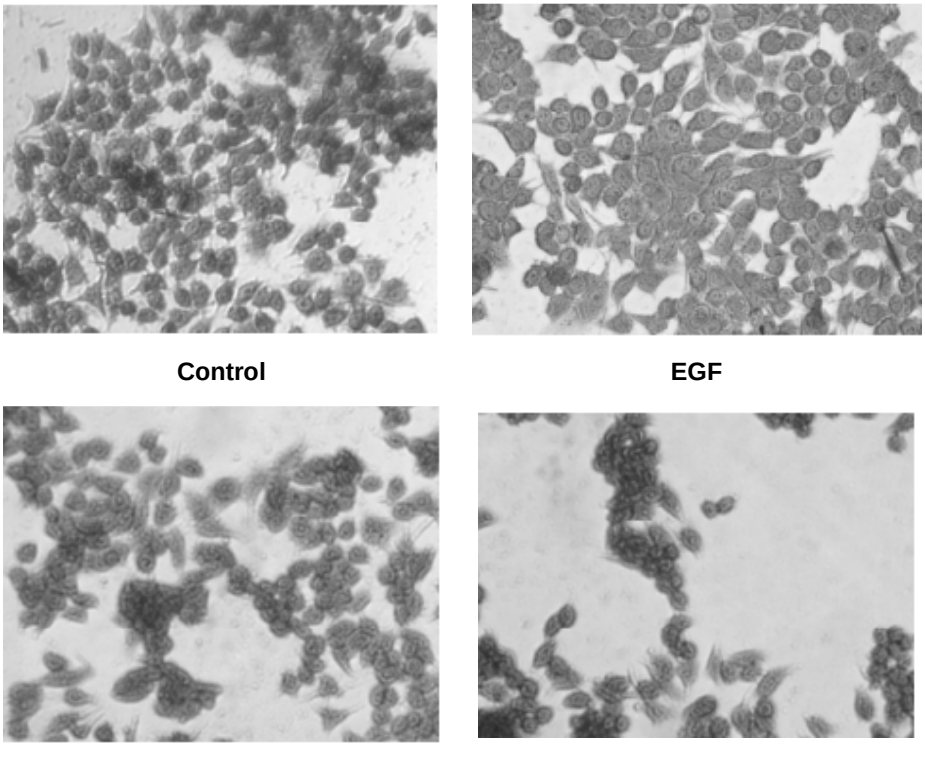

Fig. 2. Morphological features of attached to the substrate cells under influence of EGF, NTM, applied individually and in combination; crystal violet staining; x320

Table 2. Percentage of apoptotic and dead MCF-7 cells under the influence of NTM, EGF applied separately and in combination

Test samples	Content of apoptotic cells, %	Content of dead cells, %
Control	6,4±1,1	9,3±1,8
EGF	2,1±0,2*	0,5±0,0*
NTM (1,5 mg/ml)	24,5±0,3*	17,4±3,5*
NTM (1,5 mg/ml) + EGF (5 nM)	11,3±0,9* ^	34,6±2,2* ^

** ^- P<0.05, compared with control and separate using of test sample*

Obtained data on combination influence of NTM and EGF need more investigations with another cell lines with high expression of the EGFR and another experiment scheme.

The last research showed that labeled (131)I-Nimotuzumab can be used as a target drug in the high expression ot EGF-R malignancy therapy [18].

It's look like in our investigation we need to use combine simultaneous schemes of EGF+NTM influence. Because of EGF occupied homodimers of ErbB-1 are destined for rapid endocytosis and lysosomal degradation that efficiently terminate signaling. In the presence of ErbB-2 (or ErbB-3), EGF signals are enhanced because ErbB-1/ErbB-2 heterodimers release EGF when the pH of early endosomes decreases [19].

Some of authors showed that the combined application of Nimotuzumab and low-dose UV-C in vitro has an advantageous antitumor effect in salivary adenoid cystic carcinoma compared to the application of UV-C alone. NTM suppresses epithelial-mesenchimal transition and enhances apoptosis [20].

4. CONCLUSION

Synergetic cytotoxic / cytostatic effect of NTM and EGF requiring another detail research with investigations of additional cell lines and as well as experimental scheme.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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