

The effect of cetuximab on apoptosis and cell cycle phases in breast cancer cells in vitro

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SUMMARY

The aim of the study was to check how cetuximab affects the cell cycle and apoptosis of breast cancer cells.

The study was conducted on primary breast cancer cell lines and non-neoplastic cell lines derived from samples collected from patients with histopathologically diagnosed T_p1-2 N_p0 M_p0 ductal carcinoma. The effect of this chemotherapeutic agent on cells in the G₁, S, G₂/M cell cycle phases and on apoptosis of these cells was analyzed in a flow cytometer. Cetuximab, a monoclonal antibody, at a dose of 10 and 100 µg/mL of the culture medium was used for the study.

Cetuximab is capable of inducing apoptosis and inhibiting cancer cells in the S, and G₂/M cell cycle phases.

This cytostatic agent can constitute a potential strategy in phase-dependent treatment of breast cancer. However, it is not effective enough to be used in monotherapy.

Key words: breast cancer, apoptosis, cell cycle phases, cetuximab

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INTRODUCTION

Breast cancer is the most common malignancy affecting women. According to annual observations, there are almost 15,500 new cases of this disease and more than 5,200 are fatal. Breast cancer represents 22.4% of all cancers among women and 12.9% of deaths due to oncological diseases.

In the case of TNBC (triple-negative breast cancer), there is no expression of three receptor types, i.e. HER2, progesterone and estrogen receptors. This type of cancer accounts for 10–20% of invasive breast cancer cases. The prognosis is worse than that of other breast cancer types. In this case, therapy efficacy is not considerably dependent on the initial stage of the disease [1].

Cetuximab (C225) is a chimeric human-murine monoclonal antibody that binds competitively to epidermal growth factor receptor (EGFR) with high affinity, five times higher than natural ligands. The connection of cetuximab to EGFR prevents stimulation of this receptor by endogenous ligands, thereby inhibiting cell proliferation, enhancing apoptosis and reducing angiogenesis, invasion and metastasis. The influence of this antibody on the receptor results in internalization of the receptor–antibody complex that regulates EGFR expression. This receptor is currently at the center of interest in new anti-cancer therapy. Preclinical studies have shown that cetuximab reduces chemotherapy and radiotherapy resistance *in vitro* in human tumor cell lines and in nude mice.

Clinical and preclinical studies have shown that this antibody induces response to treatment in combination with chemotherapy in patients who have previously responded to chemotherapy. Based on these studies, cetuximab can be added to treatment schemes with docetaxel, cisplatin, carboplatin, irinotecan, paclitaxel and fluorouracil, thus increasing treatment efficacy [2].

Clinical studies suggest that patients with triple-negative breast cancer expressing epider-

mal growth factor receptor (EGFR) may benefit from cetuximab therapy, which targets EGF. NK cells are principal effectors of antibody-dependent cellular cytotoxicity (ADCC), and this plays a role in antibody-based therapies. Cetuximab-induced ADCC was described earlier in *in vitro* studies in patients with advanced breast cancer. Possible reasons for functional NK cell deficiency have also been investigated. Receptors activating/inactivating NK cells in peripheral blood of patients with breast cancer have been characterized and an increase in the number of CD85-inhibiting receptors has been observed. The number of NK cells needed to trigger cetuximab-mediated ADCC against TNBC cells is inversely proportional to CD85 expression, even in the presence of stimulating cytokine IL-2 or IL-15. This means that patients with high CD58 expression have an impaired TNBC cell lysis capacity in the presence of cetuximab. It has also been found that CD85 overexpression is associated with the expression of HLA-I and soluble HLA-G by tumors. The functional blockade of CD85 by its antagonist antibody reproduces ADCC levels in breast cancer patients and reintroduces this negative effect.

These data suggest that overcoming immune activation barriers can improve the clinical efficacy of cetuximab [3].

Flow cytometry is currently widely used for diagnostic purposes. It is applicable in oncology, for instance in lymphomas or leukemias, in the diagnosis of immune processes, immunodeficiency, e.g. diGeorge syndrome or AIDS, transplantation, for detection of autoimmune diseases, such as rheumatoid arthritis, Sjogren's syndrome or type 1 diabetes mellitus as well as in allergy studies [4,5].

MATERIAL AND METHODS

The study used primary cell lines derived from samples collected from women with a histological diagnosis of T2N1M0.

The study used cetuximab (Erbix Merck), a monoclonal antibody, at a dose of 10 µg and 100 µg/mL of culture medium. Cultures were grown in standard conditions: 37°C, 5% CO₂ and 90% humidity in RPM1 1640 medium supplemented with 10% fetal bovine serum (FBS) as well as penicillin and streptomycin.

Cell viability was tested using trypan blue. This compound is used for staining the cytoplasm of dead cells (apoptosis or necrosis) dark blue. Living cells remain unstained. Cell viability in cultures was over 90%, and initial density was 10⁴ cells/mL. Upon trypsinization, cell viability was determined using 0.5% aqueous solution of trypan blue. Cetuximab (Erbix Merck) was added at a concentration of 10 and 100 µg/mL to cell lines. Cells were incubated for 72 hours, and the percentage of cells in each phase and the percentage of apoptotic cells were measured in the flow cytometer Fasca.

RESULTS

The table shows the percentage of cells in individual cell cycle phases and the percentage of apoptotic cells.

DISCUSSION

The measurement with a flow cytometer showed varying numbers of cells in different cycle phases and dead cells by apoptosis in the tested cell lines treated with cetuximab in two concentrations: 10 mg and 100 mg.

When analyzing the percentage of cells in different cycle phases, it can be observed that their greatest number remained in the G1 phase. In terms of cell count, the G1 phase was followed by the G2/M phase, also for each analyzed culture. In the S phase (DNA synthesis), there were fewer cells in all the cultures.

It can be observed that the number of cells in neoplastic and non-neoplastic cultures is similar in the G1 phase. In this phase, the num-

Tab. 1.

Cell culture and cetuximab dose (µg/mL)	Cells in different cell cycle phases (%)			Apoptotic cells (%)
	G1	S	G2/M	
Breast cancer cells				
10 µg/mL	47.2	15.8	32.5	4.5
100 µg/mL	55.8*	14.1*	23.2*	6.9*
Non-tumor cells				
10 µg/mL	48.2	23.2	25.7	2.9
100 µg/mL	60.1*	17.8*	18.9*	3.2

*P < 0.05

ber of cells increased with an increase in cetuximab concentration in both cultures.

In the S phase, the number of tumor cells decreases in a statistically significant way under the influence of a higher dose of cetuximab. In tumor cell culture, the number of cells in the replication phase is lower than the number of non-neoplastic cells in the same phase of the cycle.

The G2/M phase is characterized by a statistically significant decrease in the number of neoplastic and non-neoplastic cells under the influence of both applied doses of cetuximab. A higher dose of this chemotherapeutic agent resulted in a significant reduction of cell counts in the G/M phase.

A dose of 100 $\mu\text{g}/\text{mL}$ of cetuximab induced tumor cell apoptosis.

G1 phase progression and entry into the S phase depends on the activation of cyclin-dependent kinases (CDK4/CDK6 and CDK20), which is regulated by checkpoints that control and balance extra- and intracellular factors. Transition to the S phase is regulated by growth factor expression.

Research carried out thus far reveals cetuximab-mediated cell cycle arrest in a variety of tumor cell lines [6].

Clinically, cetuximab is effective in the treatment of colorectal cancer as well as head and neck cancers.

Cetuximab binds to domain III of EGFR, thereby preventing ligand attachment, receptor dimerization and activation, thus leading to endocytosis [7]. Regulation caused by this monoclonal antibody, lysozyme-dependent EGFR degradation, supports the therapeutic effect of this cytostatic.

Roberti M.P. et al. conducted a study which found that cetuximab does not affect proliferation arrest or apoptosis induction in breast cancer IIB-BR-G and IIB-BR-G MT cell lines [6]. IIB-BR-G was obtained from invasive ductal carcinoma [7], and II-B-BR-G MT is a spontaneous form with metastases, generated in nude mice *in vivo*. To examine the direct effect of cetuximab on EGFR tyrosine kinase, the antiproliferative activity of this monoclonal antibody was first examined. For both these cultures, a moderate anti-proliferative effect of cetuximab was observed only for a concentration of 100–200 $\mu\text{g}/\text{mL}$. The concentration of 200 $\mu\text{g}/\text{mL}$ resulted in proliferation inhibition in 25 per cent. For comparison, in Caco-2 culture in colorectal cancer with positive EGFR

and KRAS wild-type, cell proliferation was inhibited in 90% with cetuximab at a concentration higher than 10 mg/mL [8].

Investigations have shown that, in triple negative breast cancer (TNBC), the antiproliferative activity or the triggering of apoptosis is relatively low or does not occur at all, even when using the highest achievable plasma dose. The inability to respond to cetuximab was associated with KRAS mutations, which lead to constitutive, mitogen-activated protein kinase signaling [9]. Nevertheless, cetuximab should be able to bind with tumor cells with mutations in the EGFR kinase domain or mutant KRAS because the extracellular portion of the EGF receptor is not covered by the modification [10]. That is why researchers have focused on the ability of this monoclonal antibody to initiate tumor-specific immune response. A new finding of these studies was that cell lines of triple-negative breast cancer (TNBC) expressing EGFR can be killed by cetuximab-induced antibody-dependent cytotoxicity (ADCC) in spite of the KRAS mutation manifested by independence from the immune mechanisms induced by cetuximab via tyrosine kinase signaling pathways [8].

The research has also shown that IL-2 and IL-15 play an important role in the ADCC induction by cetuximab. IL-2 enhances the ADCC effect, while IL-15 has a similar structure and properties to IL-2 and can replace it in a number of activities in the immune system, including induction of T-cell proliferation, NK cell growth stimulation and interferon gamma production [11,12].

Irrespective of blocking intracellular EGFR signaling, *in vitro* studies suggest that ADCC could be a useful mechanism for antitumor activity of this chemotherapeutic agent in breast cancer. The combination of this drug with immune response modulators, such as IL-2 or IL-15, may be considered an effective way to strengthen the clinical effect of cetuximab in TNBC [8].

CONCLUSIONS

Cetuximab showed the phase-dependent effect in the cell cycle. It had a positive influence on the G1 and G2/M phases by stimulating cells to remain in the G1 resting phase and significantly reducing the level of cells in the G2/M phase. A decreased number of cells in this phase attests to effective action of cetuximab in inhibiting tumor proliferation.

The antiproliferative activity of cetuximab is also reflected in its ability to reduce the number of cells in the DNA synthesis phase.

Cetuximab is capable of inducing apoptosis of cancer cells.

It may therefore constitute a potential strategy in breast cancer treatment. However, its effect is not very potent and requires a combination with other anticancer agents.

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