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Cytotoxic effect of the endothelial-monocyte activating polypeptide II on glioma cells *in vitro*

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Background. The contemporary strategies of traditional cancer treatment include surgery, radiotherapy, chemotherapy and immunotherapy. Although in recent years, chemotherapy has shown a significant increase in the effectiveness of malignant disease treatment, unfortunately, it can produce a wide range of undesirable side effects and drug resistance. Therefore, the search for novel chemotherapeutic drugs especially anticancer proteins with lower toxicity is very relevant. A tumor-derived cytokine endothelial-monocyte activating polypeptide II (EMAP II) might be helpful for this purpose. Earlier, it was shown that EMAP II inhibited endothelial cell proliferation, vasculogenesis and can induce apoptosis. **Aim.** To investigate the *in vitro* cytotoxic effect of EMAP II on glioma cells by determining the viability of cell culture using the MTT test. **Materials and Methods.** The culture of the human glioma cell line U251MG and the primary cell culture obtained from the malignant glioma tissue fragments after surgical intervention were treated with EMAP II at different concentrations. Cell viability was determined by MTT assay. **Results.** Cytokine EMAP II exhibits dose-dependent cytotoxic properties in the U251MG cell culture. In the studied concentration range (1.024 nM – 10.0 μM), it exhibits in the MTT test a biphasic effect on cell survival with two statistically significant minima in the concentration range of 640.0 pM (71.6 ± 6.4 %) and 10.0 μM (49.2 ± 11.4 %). The obtained results may indicate that EMAP II can interact with its receptors at ultralow concentrations in a different way than at high concentrations. A dose-dependent cytotoxic effect of EMAP II on glioma cells in the primary culture was shown as well but in the glioblastoma cells this dependence was less pronounced than in the gliomas grade 3 of anaplasia ones. It may be connected with both individual features of patients and genetic differences between glioblastoma and diffuse gliomas grade 3 of anaplasia cells. **Conclusions.** The complex pattern of dose dependent action of EMAP II on glioma cells may be a result of the multifunctionality of this polypeptide and its ability to interact with various target receptors.

Keywords: cytokine EMAP II, glioma cell line U251MG, primary glioma cell culture, cytotoxicity.

Introduction

The incidence of oncological diseases, including brain cancer, has increased worldwide in recent years. Gliomas comprise more than 30 percent of all brain tumors and 80 percent of all malignant brain tumors [1–3]. Nowadays, the main traditional strategies for the cancer treatment include surgery, radiotherapy, chemotherapy and immunotherapy [4]. They are used both in the majority (50–60%) of the central nervous system primary tumors separately and in combinations. The choice of the treatment method depends on many factors, such as the location and degree of anaplasia of the tumor, the general state of the patient, and so on.

Surgical removal of tumors with the aim of maximal reduction of tumor tissue is a mandatory and basic stage in the treatment tactics for malignant gliomas, but only under the condition of maintaining a high quality of life for patients [2]. If open surgery is impossible, it is necessary to be limited to a biopsy of the tumor to verify it and to determine its molecular profile. Radiation therapy and chemotherapy are usually used in an adjuvant mode, and only when surgical intervention is impossible, these methods are used as independent palliative treatment methods. Immunotherapy is a promising but little studied area of glioma treatment, since the main efforts of researchers are directed at creating dendritic cell vaccines [4].

In recent years, a significant increase in the effectiveness of malignant glioma treatment has been associated with antiblastic chemotherapy. There are several main types of contemporary chemotherapy agents based on their chemical structures and the way they affect cancer cells. Although currently, chemotherapy is an effective method of treating malignant gliomas, unfortunately, it can produce a wide range of undesirable side effects due to the damage of other proliferating cells of the body (blood, *etc.*) [5, 6]. Another problem is the drug resistance developed after long-term chemotherapy treatment [7]. Therefore, it is very important to find new less toxic therapeutic drugs for the treatment of oncological diseases.

The protein-based pharmaceuticals are among the fastest growing category of therapeutic agents in the clinic and as commercial products, typically targeting high-impact areas such as various cancers [8, 9]. Modern targeted strategies based on antitumor proteins to overcome cancer drug resistance constitute a key aim of cancer research [10, 11]. A novel endothelial-monocyte activating polypeptide II (EMAP II) may be useful for this purpose.

Multifunctional EMAP II protein has several properties: antitumor activity, inhibition of cell proliferation, promotion of apoptosis and involvement in angiogenesis and embryogenesis (Fig. 1) [12–15]. Earlier, based on animal experiments it was shown that EMAP II reveals a pronounced antitumor effect on the prostate carcinoma in a mouse xenograft model [16, 17].

In this study, we have examined the cytotoxic potential of EMAP II on both human glioma cell line U251MG cultures and primary cultures of human malignant glioma cells isolated from glial tumors during surgical operations at the Romodanov Institute Neurosurgery, National Academy of Medical Sciences of Ukraine.

Materials and Methods

Cell cultures. In this work, both the cancer glioma cell line U251MG (human glioma) and

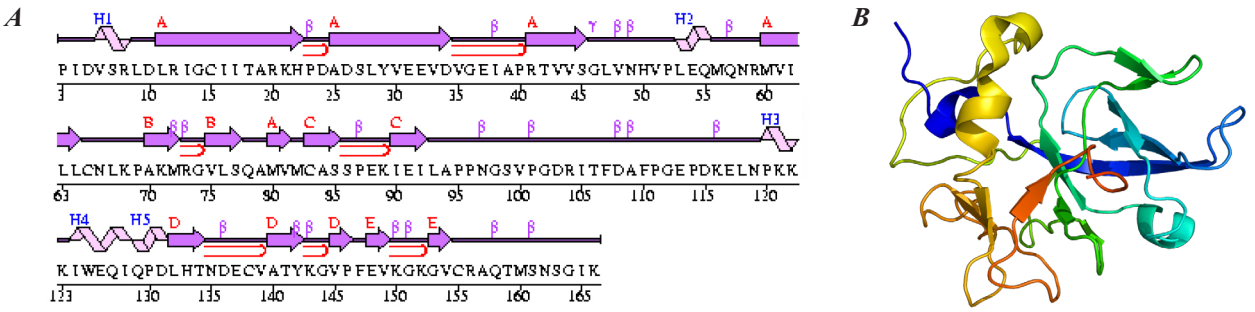

Fig. 1. Amino acids sequence and secondary structure of EMAP II (***A***), 3D crystallographic structure of EMAP II polypeptide (***B***), tRNA binding motif (Ile4-Ile105), leukocyte migration and inflammation motives (Asp5-Ala19), endothelial cells migration (Ser1-Met60) and heparin binding motives (Ala70-Met72, Pro120-Lys122) (***C***).

the primary cell lines obtained from the tissue fragments of malignant gliomas after surgical intervention were used.

The cell line U251MG was obtained from the Cell Bank of Human and Animal Tissue Lines of the Department of Experimental Cell Systems of the RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine. The primary culture of human glioma cells was prepared as described earlier [18]. The tumor fragments after surgical removal were used according to the protocol of the Commission on Ethics of the Romodanov Institute of Neurosurgery, National Academy of Medical Sciences of Ukraine.

In brief, an optimized scheme includes the following steps:

1. Obtaining a tumor fragment after surgical removal

2. Preparing the tumor fragment for work (removal of blood vessels, areas of necrosis, *etc.*)

3. Obtaining a suspension of tumor cells (5×10^9 cells/ml) in physiological solution.

4. Count the number of cells in a light microscope and a Horyaev camera with the exclusion of 0,4 % trypan blue.

Two types of samples of glioma fragments with different degrees of anaplasia: diffuse gliomas grade 3 of anaplasia and glioblastoma were studied in this research.

Recombinant EMAP II protein. The recombinant EMAP II protein was expressed in E. coli, purified and studied as described previously [19, 20]. We used the recombinant protein strain-producer derived from the E. coli *BL21(DE3) pLysE* recipient. A standard method was used to transform strain by *pET30a* EMAPII plasmid construction. A selective marker of plasmid is *kan* gene, which provides stability for the transformed cells to the kanamycin antibiotic. One

colony of strain-producer was inoculated into the Luria-Bertani (LB) medium and was ramped up overnight. LB medium contained 5 g of yeast extract, 10 g of trypton, 10 g of NaCl in 1 liter with addition of the kanamycin antibiotic to a final concentration of 30 mg/ml. Overnight culture was inoculated into fresh LB medium and cultured with intensive aeration (180 rpm) at 37 °C until it reached optical density 0.5–0.8 at 600 nm. IPTG inducer (isopropyl-β-tiohalaktopiranozyd, Sigma, USA) was added to a final concentration of 1.0 mM to induce synthesis of recombinant proteins. After induction of expression the culture was incubated for 4 hours. The recombinant proteins were obtained from lysed cell supernatants of E. coli by metal chelate affinity chromatography on Ni-NTA-agarose (Qiagen, Germany). The concentration of purified recombinant protein EMAP II was determined spectrophotometrically using extinction coefficients of 8730 M^{-1} cm^{-1} (0.23 mg/ml) for at 280 nm. The recombinant protein EMAP II was dissolved in 50 mM NaPh-buffer,150 mM NaCl, pH 7.5.

Treatments. The cells were cultivated under standard conditions in Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% fetal bovine serum and antibiotics (100 units/ml of penicillin and 100 mg/ml of streptomycin). The cells were seeded onto 96-well culture plates (5000 cells/well) and allowed to attach for 24 h in a CO_2 incubator at 37 °C with 5% CO_2. After that the medium was removed and replaced in each well with 100 µl of EMAP II in different concentrations in the serum-free DMEM growth medium. The cells were incubated with the cytokine for 24 h at 37 °C with 5% CO_2. The following protein concentrations were used: 1.024 pM, 5.12 pM, 25.6 pM, 128.0 pM, 640.0 pM, 3.2 nM, 16.0 nM, 80.0 nM, 400.0 nM, 2.0 µM, 10.0 µM. Each concentration was tested in triplicate. As control the intact cells were subjected to a similar procedure but without adding EMAP II.

Cell viability assay. The cytotoxic effect of EMAP II on glioma cells was examined by determining cell viability using the MTT test. The cell viability means the ability of cells to perform specific functions and realize their mitotic potential.

MTT test. Colorimetric test for assessing the metabolic activity of cells [21]. This test is based on the ability of a colorless tetrazolium salt MTT (3–[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in the presence of mitochondrial enzymes of living cells to be restored to insoluble formazan of a purple color, that allows estimating the number of viable cells. Briefly, 15 µl of the MTT stock solution (5 mg/ml) was added to each well and cells were incubated at 37 °C with MTT for 40 min. To dissolve the formed purple formazan 200 µl of dimethyl sulfoxide (DMSO) was added to each well. Within 5–10 minutes, formazan was dissolved in DMSO. The optical density of the formazan solution was measured at a wavelength of 570 nm using a UNIPLAN plate spectrophotometer.

The percent of cell viability was calculated as the ratio of the optical density in the experiment after EMAP II treatment to the optical density in control. The toxic effect was determined by comparing the MTT test results in the experimental and control samples.

Cell survival was determined as the percentage of living cells compared to the control.

Statistical analysis. All experiments were carried out in triplicate. Differences between

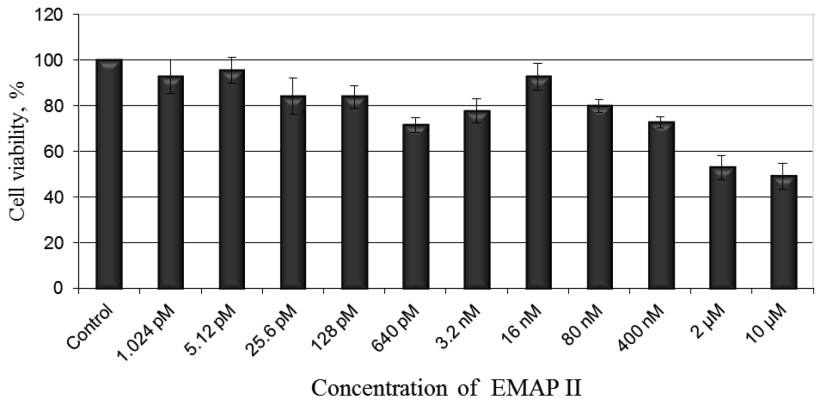

Fig. 2. Cytotoxic effect of the cytokine EMAP II at different concentrations on the U251MG glioma cell line culture. MTT test.

the control and each dose group were analyzed using Student's t-test. The mean value and the standard deviation were calculated in Excel (Microsoft, USA) program.

Results and Discussion

Nowadays, the problem of finding new chemotherapeutic drugs with low toxicity remains very important in anticancer therapy. For these purposes, the biomolecules such as proteins and peptides are of growing interest [22, 23]. One of the promising anticancer proteins is a tumor-derived cytokine EMAP II that activates host-response mechanisms. Now recombinant EMAP II is widely used in biomedical research as a cytotoxic protein [24–29]. Earlier it was shown that EMAP II exhibited significant antitumor activity to human prostate adenocarcinoma xenografts in mouse models [16, 17].

To assess the cytotoxic potential of different concentrations of EMAP II on glioma cell cultures we determined the cell viability using the MTT test. Two types of glioma cells were used, i.e. the standard cell line U251MG and the primary cell lines obtained from malignant gliomas after surgical resection. In this study we compared the cytotoxic effects of different concentrations from 1.0 pM to 10.0 μM of EMAP II cytokine on the glioma cell line U251MG (human glioma) and the primary cultures of glioma cells from tumors of specific patients using the MTT test assay.

As a result, EMAP II exhibits dose-dependent cytotoxic properties in the U251MG cell culture (Fig. 2). The cytotoxic effect of EMAP II at different concentrations on the cell culture of the glioma cell line U251MG is presented as a percentage of cell viability after EMAP II treatment compared to the intact control (Fig. 2). In the concentration range studied (1.024 pM – 10.0 μM), it exhibits a biphasic effect on cell survival with three statistically significant minima at the concentrations of 640.0 pM (71.6 ± 6.4 %), of 2.0 μM (52.2 ± 7.1 %) of and 10.0 μM (49.2 ± 11.4 %).

In the next step, we studied the primary cell lines obtained from tissue fragments of malignant gliomas after surgical intervention. Primary cultures were obtained from fragments of diffuse gliomas of grade 3 of anaplasia and glioblastomas of grade 4 anaplasia. The results of the cytotoxic effect of EMAP II on the pri-

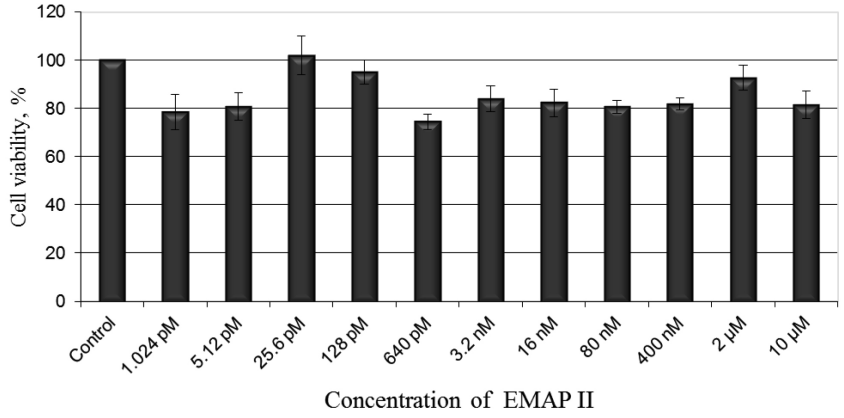

Fig. 3. Cytotoxic effect of the cytokine EMAP II different concentrations on the primary cell culture obtained from malignant gliomas tissue fragments (diffuse gliomas grade 3 of anaplasia) after surgical intervention. MTT test.

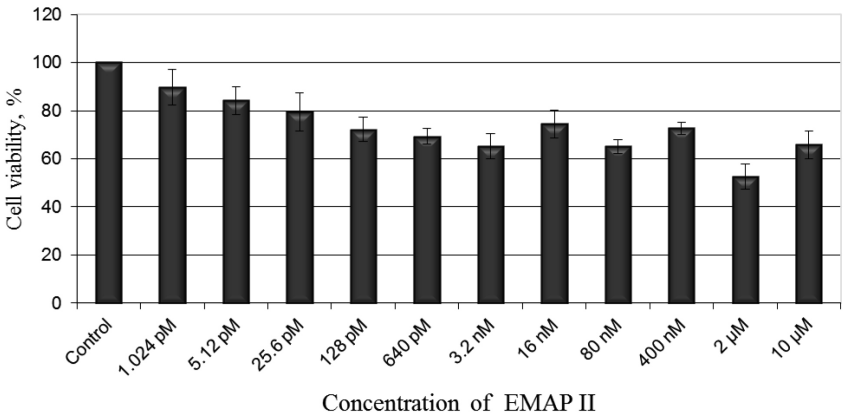

Fig. 4. Cytotoxic effect of the cytokine EMAP II different concentrations on the primary cell culture obtained from malignant gliomas tissue fragments (glioblastoma) after surgical intervention. MTT test.

mary cell culture obtained from malignant glioma tissue fragments (diffuse gliomas grade 3 of anaplasia) are presented in Fig. 3. The cytotoxic effect of EMAP II different concentrations on the primary cell culture obtained from malignant glioma tissue fragments (glioblastoma, IDH-wildtype, grade 4 of anaplasia) is shown in Fig. 4. It was shown that a dose-dependent cytotoxic effect of EMAP II on glioma cells in the primary culture of glioblastoma cells was more pronounced than in the gliomas grade 3 of anaplasia ones. It may be related both to individual features of patients' tumors and genetic differences between patients with glioblastoma and diffuse gliomas grade 3 of anaplasia cells.

The effect of EMAP II on the primary cultures of human malignant glioma cells is less effective compared with the U251MG glioma cell line culture. It is described by a curve reflecting the different effects of low and high concentrations. The complex pattern of dose dependence action of this protein on glioma cells may be a reflection of the multifunctionality of EMAP II and its ability to interact with various target receptors.

Recent studies have shown that the antiangiogenic cytokine EMAP II can impact the

interaction of the tumor necrosis factor-alpha (TNF-alpha) with the tumor vascular endothelium and improve the results of anticancer treatment [27–29]. TNF-alpha is considered to be one of the efficient vascular targeting agents but its clinical use is limited because of high side toxicity. EMAP II helps to enhance the sensitivity of tumour vasculature to the damaging activity of TNF-alpha. It is interesting to note that the non-catalytic C-terminal domain of mammalian tyrosyl-tRNA synthetase which is the homolog of EMAP II [30], also revealed the synergistic effect with TNF-alpha [31].

EMAP II has been shown to induce apoptosis in the proliferating endothelial cells and to inhibit the viability of glioma cells by inducing autophagy [32, 33].

One more important property of EMAP II in the treatment of brain tumors lies in its ability to increase the permeability of the blood brain barrier [34–36].

Conclusions

The obtained results motivate further study of the possible synergistic action of the cytokine EMAP II with anticancer chemotherapy drugs which are used in clinical practice, e.g. with temozolomide [37]. Modern nanodelivery systems based on cytokine EMAP II may be very promising for the blood-brain barrier penetration and targeting of brain tumors.

Conflict of interests

The authors of the manuscript consciously declare the absence of actual or potential conflicts of interest.

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Цитотоксична дія ендотеліального моноцит-активуючого поліпептиду II на клітини гліоми *in vitro*

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Сучасна традиційна тактика лікування раку включає хірургію, променеву терапію, хіміотерапію та імунотерапію. Хоча в останні роки хіміотерапія продемонструвала значне підвищення ефективності лікування злоякісних захворювань, на жаль, вона може викликати широкий спектр небажаних побічних ефектів, а також резистентність до ліків. З цієї причини пошук нових хіміопрепаратів з меншою токсичністю є дуже актуальним. Для цих цілей може бути корисним поліпептид II, що активує ендотеліальні моноцити, цитокін (EMAP II). Було показано, що він виявляє прозапальну та антиангіогенну активність, спрямовану на зростаючий ендотелій. **Мета.** Дослідити *in vitro* цитотоксичну дію різних концентрацій EMAP II на клітини гліоми шляхом визначення життєздатності клітинної культури за допомогою MTT тесту. **Матеріали і методи.** Культуру клітин гліоми людини лінії U251MG та первинну культуру клітин, отриману з фрагментів тканин злоякісних гліом після хірургічного втручання, обробляли різними концентраціями EMAP II. Життєздатність клітин визначали за допомогою MTT тесту. **Результати.** цитокін EMAP II проявляє дозозалежні цитотоксичні властивості в культурі клітин U251MG. У досліджуваному діапазоні концентрацій (1,024 пМ — 10,0 мкМ) він виявляє за результатами MTT тесту двофазний ефект на виживання клітин з двома статистично значущими мінімумами в діапазоні концентрацій 640,0 пМ (71,6 ± 6,4 %) і 10,0 мкМ (49,2 ± 11,4 %). Отримані результати можуть свідчити про те, що EMAP II у наднизьких концентраціях може взаємодіяти з рецепторами інакше, ніж при високих концентраціях. Було також показано дозозалежний цитотоксичний ефект EMAP II на клітини гліоми в первинній культурі, але в клітинах гліобластоми ця залежність була менш вираженою, ніж у гліомах 3 ступеня анаплазії. Це може бути пов›язано як з індивідуальними особливостями хворих, так і з генетичними відмінностями між гліобластомою та дифузними гліомами 3 ступеня анаплазії. **Висновки.** Складний патерн дозозалежної дії цього білка на клітини гліоми може бути результатом багатофункціональності EMAP II та його здатності взаємодіяти з різними цільовими рецепторами.

Ключові слова: цитокін EMAP II, стандартна лінія клітин гліоми U251MG, первинна культура клітин гліом, цитотоксичність.

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