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NANOCOMPOSITE COMPLEX EMAP II INFLUENCE ON TUMOR NECROSIS FACTOR AND INTERFERON

L. A. Kolomiets-Babenko[1, 2]
O. S. Bohorad-Kobelska[3]
N. L. Kovalchuk[2]
M. Ja. Spivak[3]
A. I. Kornelyuk[1]

[1]Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine, Kyiv
[2]Taras Shevchenko National University of Kyiv, Ukraine
[3]Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv

E-mail: babenko_lesia@ukr.net

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The goal of the research was to determine the ability of new nanocomposite preparation EMAP II (endothelial monocyte activating poplypeptide II) to affect the expression of the tumor-necrosis factor and interferon *in vitro*. In the experiments, the transformed cell line L929 cells was used. The induced interferon levels were determined in samples of culture medium by the microtitration method in the L929 cell culture against test virus vesicular stomatitus VSV. Toxicity of the substance was assessed by its maximum tolerated dose. The amount of endotoxins in nanocomposite preparation EMAP II was measured using gel-clot test. The range of concentrations of EMAP II causing the production of tumor necrosis factor was determined. The concentration of lipopolysaccharides in the tested nanocomposite preparation was less then 0.5 IEU/kg. New nanocomposite preparation EMAP II has the ability to induce TNF-α production at rather low concentration 1.56–25.00 μg/ml (82.49–1370.00 mol × 10^{-12}). The interferon production under the influence of nanocomposite preparation EMAP II was not found. The results support the application of the target nanocomposite preparation EMAP II for cancer treatment.

Key words: nanocompomposite EMAP II, tumor-necrosis factor TNF-α, interferon

Designing of the nanotechnologies to produce new highly efficient drugs based on biocompatible polymers is important for the development of personalized treatment of cancer patients. Hence it is obvious that the new pharmaceuticals for clinical oncology characterized by low toxicity including the studied nanocomposite preparation EMAP II (endothelial monocyte activating polypeptide II), may target both domestic and foreign markets.

In the Institute of Molecular Biology and Genetics of the NAS of Ukraine were developed scientific and methodological basis for the creation of bionanosystems for controlled delivery of anti-angiogenic cytokine EMAP II. The EMAP II bacterial expression system in *E. coli* BL21 (DE3) cells was optimized, and highly purified cytokine preparation EMAP II was isolated in preparative quantities using biotechnological methods [1]. The next goal was to develop a nanocomposite anticancer preparation which would allow the controlled introduction to neoplastic cells, thus reducing the effective doses of EMAP II, to achieve positive results and to increase its efficiency and stability. The combination of components and their relationship was chosen using bio-informative analysis. The developed bionanosystems will be used for delivering anti-angiogenic EMAP II cytokine to endothelial cells for nano-treatment of tumors. Dextran-70 is used as polymer coating, cryoprotectant and filler of the nanocomposite preparation, as a stabilizer reducing the damaging effect of freeze-drying, and providing controlled delivery of the drug to the tumor cells, thereby increasing the activity of the drug [2].

EMAP II is expressed in the brain, intestine, thymus, lungs, endocrine glands, nervous and other tissues. EMAP II expression in tissues increases if they are damaged, because EMAP II is involved in inflammatory

reactions, is able to improve the sensitivity of tissues to tumor necrosis factor (TNF-α), to attract neutrophils and lymphocytes to the site of injury. Endogenous EMAP II enhances antitumor properties of TNF-α in the tumor microenvironment.

TNF-α is an endotoxin-induced protein or kachectin with necrotizing action, synthesized by activated macrophages in the early stages of inflammation [3, 4]. TNF-α is a primary mediator in the pathogenesis of bacterial, viral, and fungal infections. Reduction of TNF-α in blood in case of infection indicates the failure of the body's protection system [5]. It activates phagocytes, neutrophils, promotes the growth and differentiation of endothelial cells. TNF-α has immuno-modulating and inflammatory effects, cytostatic and cytolytic activity against certain tumors, enhances the growth of blood vessels in normal and suppress in tumor tissues [6]. Tumor necrosis factor invokes focused immune response, attracting inflammatory cells [7].

The production of tumor necrosis factor, in turn, can influence interferon, a protein known for modulating immune response, which among other properties also prevents the development of cancer.

Since the antitumor effects of the EMAP II preparation are closely related to tumor necrosis factor, it was important to determine its concentrations, necessary for the production of TNF-α, and also identify possible impact on the production of interferon.

The aim of the study was to create a more stable preparation of EMAP II complex with dextran. It is the generally accepted approach of developing therapeutic agents based on proteins, as they are labile in an isolated state and can be deactivated because of proteolytic degradation, denaturation or aggregation. It was also important to prove that dextran does not affect the studied properties of EMAP II cytokine. Below is a comparison of separate performances of the EMAP II preparation and dextran in appropriate concentrations.

One of the conditions for the controlled delivery of the EMAP cytokine is high protein stability in the formed complex that makes the delivery of the cytokine to the tumor cells more efficient. In addition, it is known that polysaccharides can interact with co-receptors in the cytokine-receptor complex. The physiological effect of EMAP II cytokine is due to the interaction with the respective receptors exhibited on the surface of tumor cells, but not, probably, on the surface of intact cells.

Materials and Methods

Reagents. In this work, nanocomposite preparation EMAP II with dextran-70 was used, allowing to increase the activity and stability of the therapeutic protein and thereby reducing the effective dose to achieve the positive result. The preparation contained EMAP II, buffer: 50 mmol PBS, pH 8.0; 150 mmol NaCl, and dextran-70. To prepare the base solution, lyophilized nanocomposite preparation was dissolved in distilled water to a concentration of 0.1 mg/ml of the target protein. The investigational preparation is called nanocomposite because the cytokine EMAP II itself is a nanoscale, as its diameter is about 30 nm. The physiological effect is caused by the interaction of EMAP II with the receptor, the cytokine being in conjunction with dextran-70, the stabilizer of protein conformation. The dextran-70 is also a cryoprotectant as a part of lyophilized preparation inhibiting the aggregation of protein. In the nanocomposite complex, the proteins interact with the polymer matrix not at the macro- (as in the case of composite materials) but at the molecular level.

Preparations containing proteins as active ingredients are used mainly in the lyophilized form. The process of freeze-drying can damage the structure of macromolecules, and reduction or even disappearance of activity. To reduce the damaging effects, we used a polysaccharide dextran-70 as the stabilizer of the complex, cryoprotectant and filler, because it is widely used in the production of medicine drugs and is non-toxic.

The essence of the proposed EMAP II preparation is a combination of these two components. The dextran-70 allows enhancing the stability of the therapeutic protein EMAP II. Dextran-70 as part of nanocomposite preparation (in an amount of 1.5%) is the stabilizer, coating and cryoprotectant, and it reduces the possible damaging effect of freeze-drying in a nanocomposite anticancer drug. The composition of a single dose of the preparation: target protein: 0.1 mg; bufer: 50 mmol PBS; pH 8.0; 150 mmol NaCl, 15 mg±0.1% dextran-70. The amount of protein in the product after lyophilization is 100 μg per vial.

Cell lines and culture conditions. In experiments were used two transformed cell lines: L929 (fibroblasts from the connective tissue of a C3H/An mouse, subline A), obtained from the Bank of the Cell Lines of the Kavetsky

Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine, and PST (primary swine testicles) obtained from the collection of the Institute of Veterinary Medicine, UAAS.

The cells were grown in monolayer culture in glass flask in the nutrient medium 199 with 0.68 mmol L-glutamine (SPE "Bio-Test Laboratory", Kyiv, Ukraine) supplemented with 10% foetal bovine serum ("Sigma", USA), 25 mmol HEPES (pH 7.4; "Serva", Germany) and 1 μg/ml kanamycin at 37 °C, under a constant level of CO_2 (5%). The cells were reseeded in dilutions of 1:3–1:10, using 0.02% EDTA every 2–3 days. The optimal cell density during reseeding was $1–3\times10^4$ cells/cm^2.

To study the cytotoxicity and the ability to produce interferon (IFN) and TNF-α, were selected cells at the logarithmic growth phase. The cells were removed from the surface of the flasks using 0.02% EDTA, re-suspended in culture medium to a concentration of 5×10^4 cells/ml. Cell suspension was then introduced into 96-well plates ("Sarstedt", Germany) at 100 μl per well, and incubated for 24 hours in an incubator with a constant level of CO_2 (5%) at 37 °C to form the full cell monolayer.

Evaluation of toxicity. The toxicity of EMAP II nanocomposite preparation and dextran-70 was evaluated in cell lines L929 and PST. The toxicity of the substances was assessed by such indicators as: MTD (maximum tolerated dose) and CC_{50} (cytotoxic concentration that causes the death of 50% of viable cells). As MTD was taken the concentration of substance which caused the death of no more than 10% of cells compared to control. CC_{50} was the cytotoxic concentration of preparation that caused 50% destruction of the cellular monolayer. Nanocomposite preparation EMAP II in a concentration of 100 μg/ml and dextran-70 in a concentration of 354 μg/ml were added to the cellular monolayer with subsequent serial twofold dilution, and then the cells were grown for 24 h at 37 °C. In order to assess the number of cells with damaged membranes in control and after incubation with the experimental substances, 0.5% solution of trypan blue was added to the cellular monolayer.

Determination of IFN and TNF-α. The titer of interferon induced by EMAP II preparation was determined by microtitration samples in L929 cell culture against the 100 $CCID_{50}$ (cell culture infectious dose that causes the 50% lesion of the cellular monolayer) of test virus — vesicular stomatitis virus (VSV). To achieve induction of IFN, L929 and PST cells were cultured in the presence of 0.188; 0.375; 0.75; 1.5; 3; 6; 12.5 and 25 μg/ml of EMAP II preparation and corresponding concentrations of dextran-70 (from 2.77 to 354 μg/ml with consequent twofold dilution) for 24 hours. For induction of TNF-α, PST cells were used. Samples of the culture medium were collected and stored at −40 °C until determination of IFN titer and activity of TNF-α.

The level of induced IFN in samples of culture medium was determined by the microtitration method in the L929 cell culture against VSV virus (Indiana serotype, Depositary of Zabolotny Institute of Microbiology and Virology NAS of Ukraine) [8].

In 96-well plates with the formed 1-day monolayer of L929 cells, were added the samples of culture medium in a ratio of 1:8 with consecutive double dilution (final dilution 1:64). After 24 hours, the cells were treated with the VSV at a dose of 100 $CCID_{50}$. The IFN titer value was calculated as inverse to maximum dilution of the preparation which protected 50% of cells in the monolayer against the cytopathic effect of the virus, and expressed as $\log_2(\text{dulition})^{-1}$.

The biological activity of TNF-α in the culture medium of L929 cells was assessed by cytotoxic effect on the cell culture, expressed as cytotoxicity index (%) and in terms of ng/ml. Recalculation into ng/ml was performed as described in [9], using the approximation formula $Y = 3.985 \ln(X) + 75.341$, hence $X = \exp((Y-75.341)/3.985)$.

To determine the activity of TNF-α, samples of culture medium of PST cells and nanocomposite preparation EMAP II were added in ratios from 1:8 to 1:64 in the culture medium to the formed 1-day monolayer of L929 cells. Then, actinomycin D with a final concentration of 1 μg/ml was added to all wells besides the control wells. Wells with the nutrient medium were used as cell control cells and wells with actinomycin D but without samples of the culture medium were used as control for actinomycin D. The plates were incubated at 37 °C for 24 hours in humid atmosphere containing 5% CO_2. Then the nutrient medium was removed. To determine the cytotoxic effect on L929 cells, 0.2% solution of crystal violet was added to the wells. The results were assessed using microplate spectrophotometer Multiscan Ascent ("Thermo Labsystems", Finland) at 540 nm. Cytotoxicity index was calculated as $CI = (a - b)/a \times 100\%$, where a and b are

the values of performance optical density of control cells and of cells treated with samples of culture medium, respectively. The experiments were repeated 3 times.

The content of endotoxins. Endotoxins originating from Gram-negative bacteria are the most common cause of toxic pyrogenic reactions in cases of contamination of drugs. These endotoxins are lipopolysaccharides. Evaluation of the amount of endotoxins in nanocomposite preparation EMAP II was performed by gel-clot test according to the State Pharmacopoeia of Ukraine. Maximum allowable dose of endotoxin for the preparation is 5 IEU/kg. The sensitivity of LAL-reagent is 0.03 IEU.

Statistical analysis of the results. The null hypothesis for the compared control and experimental groups was assessed using the nonparametric Mann-Whitney test. Differences between the groups were considered statistically significant at $P < 0.05$.

Results and Discussion

Toxicity of nanocomposite preparation EMAP II and dextran-70

As shown in the Table 1, MTD and CC_{50} for EMAP II preparation in both cell lines are 25.00 and 50.00 μg/ml, respectively. The sensitivity of cells to dextran-70 is not equal for the different cell lines. The dose of CC_{50} to dextran-70 was not defined since 50% destruction of cellular monolayer was not observed. The given data shows that toxicity of EMAP II preparation is significantly lower than that of dextran-70.

Production of IFN under the EMAP II preparation

Production of IFN was not observed in case of introduction of the preparation EMAP II and dextran-70.

Production of TNF-α under the nanocomposite preparation EMAP II

The established range of concentrations of the EMAP II preparation which caused the production of TNF-α by the cell culture (Table 2) was 1.56–25.00 μg/ml (82.49 mol × 10^{-12}–1370 mol × 10^{-12}).

According to the results, dextran-70 has not shown the ability to influence the production of TNF-α.

Thus, the production of TNF-α under nanocomposite preparation EMAP II in concentration of 1.56–25.00 μg/ml was significantly higher than TNF-α production under the influence of dextran-70, and the difference is significant ($P < 0.05$). The concentration of dextran-70 corresponds to its concentration in the nanocomposite preparation EMAP II.

Table 1. **Toxicity of nanocomposite preparation EMAP II and dextran-70 *in vitro***

Cell line	EMAP II preparation		Dextran-70
	MTD, μg/ml (mol × 10^{-9})	CC_{50}, μg/ml (mol × 10^{-9})	MTD, μg/ml
L929	25.00 (1.37)	50.00 (2.75)	177.00*
PST	25.00 (1.37)	50.00 (2.75)	88.50*

* — $P < 0.05$ compared to EMAP II preparation.

Table 2. **Production of TNF-α under EMAP II nanocomposite preparation**

Concentration of EMAP II preparation, μg/ml	Cytotoxicity index, %	Concentration of TNF-α, ng/ml
0.19	2.83	1.25×10^{-8}
0.39	12.61	1.46×10^{-7}
0.78	13.72	1.92×10^{-7}
1.56	32.00	1.89×10^{-5}
3.13	76.46	1.32
6.25	76.16	1.23
12.50	69.55	0.23
25.00	46.71	0.76×10^{-3}
50.00	5.22	2.28×10^{-8}

According to the data presented in Table 2, TNF-α production in samples of PST cell culture medium treated with EMAP II preparation was due to cytokine EMAP II, not because of dextran-70.

Analysis of the nanocomposite preparation showed that they contained less than 0.5 IEU/kg but more than 0.03 IEU/kg of lipopolysaccharides. The maximum concentration of lipopolysaccharides allowed by State Pharmacopoeia of Ukraine is 5 IEU/kg. Thus, the observed concentration of lipopolysaccharides does not affect the production of cytokines.

It can be concluded that the nanocomposite preparation EMAP II is able to cause the production of TNF-α at rather low concentrations 1.56–25.00 μg/ml (82.49–1370.00 mol $\times 10^{-12}$). Production of interferon under the influence of nanocomposite preparation EMAP II was not found. Dextran-70 serves as a polymer coating, thereby increasing the activity of the EMAP II preparation, but does not affect the production of TNF-α.

For the first time, EMAP II is proved to be one of the factors able to modulate the response of endothelial cells to tumor necrosis factor TNF-α. The introduction of TNF-α in the blood circulatory system selectively causes thrombosis of the microcirculation of some tumors by increasing the concentration of tissue thromboplastin factor at the endothelial membranes leading to tumor necrosis [10]. Thus, the use of the EMAP II preparation is an essential element in the treatment of various tumors due to the increased influence of TNF-α on tumors.

TNF-α also induces apoptosis of activated cells and is involved in the recovery of tissues, damaged due to inflammation. A high dose of TNF-α kills the tumor vascular system and increases the absorption of preparations by the tumor. Therefore the presence of as many TNF-receptors as possible in the neoplastic endothelium is of utmost importance.

EMAP II is correlated with sensitivity of tumors to TNF-α. Based on increasing TNF-α concentration, high levels of EMAP II must be reached before treatment with tumor necrosis factor [11] because TNF action on tumor endothelial cells leads to massive thrombosis at the site of vascular injury. Experiments have shown that EMAP II can induce sensitivity to TNF in TNF-resistant tumors. Increased regulation of TNF leads to growing procoagulant activity as well as the permeability of the vascular system.

These data support the use of target nanocomposite preparation of EMAP II in the treatment of cancer patients.

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ВПЛИВ НАНОКОМПОЗИТНОГО ПРЕПАРАТУ ЕМАР ІІ НА ПРОДУКЦІЮ ФАКТОРА НЕКРОЗУ ПУХЛИН ТА ИНТЕРФЕРОНА *in vitro*

Л. А. Коломієць-Бабенко[1, 2]
О. С. Богорад-Кобельська[3]
Н. Л. Ковальчук[2]
М. Я. Співак[3]
О. І. Корнелюк[1]

[1]Інститут молекулярної біології і генетики НАН України, Київ
[2]Київський національний університет імені Тараса Шевченка, Україна
[3]Інститут мікробіології та вірусології ім. Д. К. Заболотного НАН України, Київ

E-mail: babenko_lesia@ukr.net

Метою роботи було встановити здатність нового нанокомпозитного препарату ЕМАР ІІ (ендотеліальний моноцитактивувальний поліпептид ІІ) впливати на експресію фактора некрозу пухлин та інтерферону *in vitro*. В експериментах використовували перевивні лінії клітин L929. Визначення рівня індукованого інтерферону у зразках середовища культивування проводили за допомогою методу мікротитрування на культурі клітин L929 проти тест-вірусу — везикулярного стоматиту. Токсичність речовини оцінювали за величиною її максимально витримуваної концентрації. Кількість ендотоксинів у нанокомпозитному препараті ЕМАР ІІ обчислювали методом гель-тромб-тесту. Встановлено діапазон концентрацій препарату, за яких відбувається продукування фактора некрозу пухлин. Під час перевірки нанокомпозитного препарату на наявність ліпополісахаридів встановлено, що вміст їх — менше 0,5 МОЕ/кг. Новий нанокомпозитний препарат ЕМАР ІІ здатен спричинити продукування фактора некрозу пухлин TNF-α за низьких концентрацій 1,56–25,00 мкг/мл (82,49–1370,00 Моль×10^{-12}). Продукування інтерферону під впливом нанокомпозитного препарату ЕМАР ІІ виявлено не було. Отримані дані свідчать про перспективність використання цього препарату для лікування онкохворих.

Ключові слова: нанокомпозит ЕМАР ІІ, фактор некрозу пухлин — TNF-α, інтерферон.

ВЛИЯНИЕ НАНОКОМПОЗИТНОГО ПРЕПАРАТА ЕМАР ІІ НА ПРОДУКЦИЮ ФАКТОРА НЕКРОЗА ОПУХОЛЕЙ И ИНТЕРФЕРОНА *in vitro*

Л. А. Коломиец-Бабенко[1, 2]
Е. С. Богорад-Кобельская[3]
Н. Л. Ковальчук[2]
Н. Я. Спивак[3]
А. И. Корнелюк[1]

[1]Институт молекулярной биологии и генетики НАН Украины, Киев
[2]Киевский национальный университет имени Тараса Шевченко, Украина
[3]Институт микробиологии и вирусологии им. Д. К. Заболотного НАН Украины, Киев

E-mail: babenko_lesia@ukr.net

Целью работы было установить способность нового нанокомпозитного препарата ЕМАР ІІ (эндотелиальный моноцитактивирующий полипептид ІІ) влиять на экспрессию фактора некроза опухолей и интерферона *in vitro*. В экспериментах использовали перевивные линии клеток L929. Определение уровня индуцированного интерферона в образцах среды культивирования проводили с помощью метода микротитрования на культуре клеток L929 против тест-вируса — везикулярного стоматита. Токсичность вещества определяли по величине ее максимально выдерживаемой концентрации. Количество эндотоксинов в нанокомпозитном препарате ЕМАР ІІ вычисляли методом гель-тромб-теста. Установлен диапазон концентраций препарата, при которых происходит продуцирование фактора некроза опухолей. При проверке нанокомпозитного препарата на наличие липополисахаридов установлено, что их содержание меньше, чем 0,5 МОЕ/кг. Новый нанокомпозитный препарат ЕМАР ІІ способен вызвать продуцирование фактора некроза опухолей TNF-α при низких концентрациях 1,56–25,00 мкг/мл (82,49–1370,00 Моль×10^{-12}). Продуцирование интерферона под влиянием нанокомпозитного препарата ЕМАР ІІ обнаружено не было. Полученные данные свидетельствуют о перспективности использования этого препарата для лечения онкобольных.

Ключевые слова: нанокомпозит ЕМАР ІІ, фактор некроза опухлей — TNF-α, интерферон.