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ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF ANTI-HUMAN HB-EGF NEUTRALIZING POLYCLONAL ANTIBODIES *IN VITRO*

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Background: **Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the epidermal growth factor family and has a variety of physiological and pathophysiological functions. Also, HB-EGF plays a pivotal role in progression of different tumors. So, HB-EGF seems to be a target molecule for the treatment of some cancer types.** ***Aim:*** **To obtain HB-EGF neutralizing polyclonal antibodies and test their anti-proliferative properties *in vitro*.** ***Materials and Methods:*** **Lab rabbits and mice were used for immunization with recombinant HB-EGF. The effect of generated polyclonal antibodies on viability and apoptosis of human epidermoid carcinoma derived A431 cell line was assessed using MTT and Annexin V-propidium iodide assays.** ***Results:*** **Rabbit polyclonal anti-HB-EGF serum could block binding of soluble HB-EGF to epidermal growth factor receptor/human epidermal growth factor receptor. Also, anti-HB-EGF antibodies could bind to surface of A431 cells which express abnormally high levels of membrane bound proHB-EGF and its receptor. It has been shown that immune serum with polyclonal antibodies against HB-EGF was able to block the mitogenic activation of the cells with HB-EGF and cause apoptotic cell death.** ***Conclusion:*** **Inhibition of HB-EGF activity with neutralizing polyclonal antibodies can effectively inhibit mitogenic activation and cause apoptosis of cancer cells with significant epidermal growth factor receptor overexpression.**

Key Words: **HB-EGF, mitogenic activation, antibodies, cancer treatment, receptors.**

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of epidermal growth factor (EGF) family of growth factors. Originally it was found to be a potent mitogen for fibroblasts and smooth muscle cells [1]. It is synthesized in a transmembrane pro-form (proHB-EGF) composed of a signal peptide, pro-peptide, heparin-binding, EGF-like, juxtamembrane, transmembrane and cytoplasmic domains [2]. ProHB-EGF can be proteolytically cleaved by matrix metalloproteinases with a formation of the soluble form (sHB-EGF) that can bind and activate epidermal growth factor receptors 1 and 4 (EGFR/ErbB1 and ErbB4) in autocrine and/or paracrine mode. This ligand binding event causes a conformational change in the structure of the monomeric receptor, promotes its dimerization and increases the tyrosine kinase activity of the intracellular domains. Subsequent autophosphorylation of specific tyrosine sites mediates activation of phosphatidylinositol 3-kinase/Akt, the Ras/Raf/MEK/ERK1/2 pathway or other signaling pathways [3]. Membrane-anchored form of HB-EGF retains the ability to signal in juxtacrine mode by interacting with receptors on a neighboring cell [4] but also can act as a receptor by itself. ProHB-EGF can bind diphtheria toxin (DT) — a major factor of pathogenicity of the *Corynebacterium diphtheriae* and facilitate its intracellular transport [5].

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Abbreviations used: DT – diphtheria toxin; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; HB-EGF – heparin binding EGF like growth factor; mAbs – monoclonal antibodies; pAbs – polyclonal antibodies; PBS – phosphate buffered saline; PI – propidium iodide; proHB-EGF – pro-form HB-EGF; sHB-EGF – soluble HB-EGF.

HB-EGF is one of the important mediators of connective tissue regeneration, heart development and blastocyst implantation [6–8]. However, most concerns are related to the growth-promoting function of HB-EGF in oncogenic transformation. This mitogen participates in tumorigenesis through stimulation of cancer cell proliferation, migration, and survival [9]. HB-EGF is highly expressed in certain cancer cells, such as ovarian and breast cancers that makes it beneficial for the development of antitumor therapeutic agents [10, 11].

Over the past years, several HB-EGF-targeted anticancer strategies were investigated. HB-EGF binding peptides showed suppression of migration and invasion of ovarian cancer cell lines by attenuation of EGFR activation, but this therapy wasn't tested *in vivo* [12]. The nontoxic mutant of DT — CRM197 — was probed on cancer models and demonstrated significant antitumor effect alone and in the combination with paclitaxel [13–16]. However, acquired immunity to diphtheria toxoid due to worldwide anti-diphtheria immunization program can be considered as one of the biggest obstacles on the way of implementation of CRM197 for clinical usage.

Another promising cancer-treatment approach with specific mechanisms of action includes monoclonal antibodies (mAbs). Some studies report about a generation of anti-HB-EGF mAbs with different epitopes and blocking activities respectively [17–22]. Some of them efficiently inhibited ectodomain shedding or receptor interaction and showed an antiproliferative effect on different cancer cell lines. However, there is no evidence about HB-EGF inhibition effect on aggressive cancer cell lines with overexpression of both growth factor and EGFR. Also, complete neutralization of HB-EGF activity

cannot be achieved by using single mAb and outcome of cumulative action of various Abs wasn't assessed.

This research aims to generate polyclonal antibodies (pAbs) to sHB-EGF and examine their ability to inhibit growth of cancer cells *in vitro*. In our study we chose pAbs because they recognize multiple epitopes on an antigen. Also, pAbs are more specific to antigen and can be generated more easily. They are tolerant for antigen polymorphism, changes in glycosylation or conformation and are robust and more stable over a broader range of pH and buffer compositions compared to mAbs [23]. Thus, pAbs could be considered as a good prototype of biopharmaceuticals for sHB-EGF inhibition and perfect tools for studying growth factor functions.

MATERIALS AND METHODS

Animals and cell lines. 4-month male rabbits (3.0 to 3.5 kg of body weight) and BALB/c female white mice (30–35 g of body weight) were used in the experiments. All animals had unlimited access to animal chow and tap water throughout the investigation. The experiment was carried out in accordance with the requirements of Bioethical Commission of the Palladin Institute of Biochemistry of the NAS of Ukraine.

A431 cells were grown in RPMI-1640 media with 10% fetal bovine serum, 0.3 g/L L-glutamine and antibiotics: 100 mg/L streptomycin, 10,000 U/L penicillin G and 250 mg/L amphotericin B at 37 °C in 5% CO_2 atmosphere.

Immunization of experimental animals. Expression and purification of recombinant sHB-EGF (rsHB-EGF) that was subsequently used for immunization of experimental animals was performed by scientific and production enterprise "Enamine" (Kyiv, Ukraine). Primary and booster injections of antigen were intramuscular, 1 site, and subcutaneous, 2–3 sites, for rabbits and intraperitoneal for mice as emulsions in Complete Freund's Adjuvant (1^{st}) or Incomplete Freund's Adjuvant (2^{nd}, 3^{rd}) administered at a dose of 100 μg per rabbit and 25 μg per mouse with 2-week intervals. The level of specific antibodies to sHB-EGF antigen in the serum of immunized animals was determined by ELISA after 7^{th} day since last immunization.

Preparation of immune serum. The blood was taken from the rabbit's ear vein or from the caudal vein of the mice. For serum obtaining, blood without anticoagulants was incubated for 40 min at room temperature and centrifuged for 30 min at 2700 rpm. Before use in tests, blood serum was heated for 30 min at a 56 °C to inactivate the thermolabile complement system, which is an important element of natural immunity and can affect the results of the proliferative test.

Recombinant protein expression. Bacterial cultures with recombinant *E. coli* cells were grown at 37 °C under aeration conditions (250 rpm) in the lysogeny broth medium with 50 mg/L of kanamycin, 170 mg/L of chloramphenicol and 1% glucose. When bacterial density reached OD_{600} — 0.3–0.5, cells were transferred to fresh lysogeny broth medium with kanamycin (50 μg/ml) and an inducer of expression of isopropyl β-D-1-thiogalactopyranoside at a concentration of 1 mM. Target proteins were expressed for 3–4 h at 30 °C under strong aeration conditions and pelleted by centrifugation at 3300 g for 15 min. Human and mouse rsHB-EGF and human proHB-EGF were purified as described in [24]. Fluorescent derivative of rsHB-EGF (mCherry-rsHB-EGF) was prepared and purified as mentioned in [25].

Enzyme-linked immunosorbent assay (ELISA). The antigens were added into the wells of the 96-well plate at a final concentration of 5 μg/mL per well in phosphate buffered saline (PBS) and left overnight at 4 °C. Non-fat 1% milk in PBS was used as a blocking agent. The incubation with pAbs against sHB-EGF in PBS-T (PBS with Tween-20 in a final concentration of 0.04%) was performed for 1 h. Animals' serum samples were diluted at a start ratio of 1:100. Anti-mouse-(1:12,000) or anti-rabbit-HRP conjugate (1:10,000) (both from Sigma-Aldrich, USA) were used for primary antibodies detection. 3,3′,5,5′-tetramethylbenzidine was used as chromogenic substrate. The color reaction was quantified by measuring the absorbance at 490 nm.

Proliferative test. 100 μL of suspension with 25,000 cells in RPMI-1640 containing L-glutamine, 10% fetal bovine serum and antibiotics: streptomycin (100 mg/L), penicillin (10,000 U/L) and amphotericin B (250 μg/L) were added to the wells of the 96-well plate. After 24 h, the wells with attached cells were washed twice with PBS and filled with a fresh fetal bovine serum-free culture medium. Control substances were administered at the following concentrations: LPS — 500 ng/mL, HB-EGF — 500 ng/mL, ionomycin — 6 μM. Serum from rabbits after heat inactivation was added at dilution 1:100. The plate was incubated for 60 h at 37 °C in 5% CO_2 atmosphere. After incubation, the contents of the wells were removed (without washing with PBS), the MTT-reagent (0.5 mg/mL) was added for 3 h.

Then 100 μl of 10% SDS in dimethyl sulfoxide acidified with 0.04 M HCl were added to the cells and incubated for 15 min at 37 °C under active stirring. After dissolving crystals, the optical density was measured at 570 nm (reference wavelength — 620 nm).

Confocal microscopy. A431 cells were grown on glass slides to 80% confluence and washed twice with 1 mL PBS containing 1% bovine serum albumin (BSA). Rabbit pAbs against sHB-EGF (1:100) or serum of the same rabbit obtained before immunization (1:100) were added to cells and incubated at 4 °C for 1 h. Glass slides were washed with PBS, anti-rabbit FITC-labeled antibodies (1:100) were added and incubated under the same conditions. Cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich, USA). Slides were mounted in DABCO/PVA mounting medium (Sigma-Aldrich, USA) and analyzed using LSM 510 META laser scanning confocal microscope (Carl Zeiss, Germany). Image analysis was performed with FIJI software [26].

Flow cytometry. The fluorescence intensity of cells was measured by Coulter Epics XL flow cytometer (Beckman Coulter, USA). A431 cells have been detached from flask by addition of 30 mM ethylenedi-

aminetetraacetic acid in PBS. Optimal quantity of cells for staining was $0.5 \cdot 10^6$ per probe. Cells have been stained with mCherry and mCherry-rsHB-EGF (10 μg/mL) proteins and serum of immunized or not-immunized rabbits in 1:100 or 1:500 dilution in 200 μL of BSA/PBS solution (1% BSA in PBS) for 30 min at 4 °C. Cell death assay was done with Annexin V — EGFP and propidium iodide (PI) as described above. For washing of non-bound proteins or fluorochromes, 1 ml of BSA/PBS was added, and then cell pellet was carefully resuspended and centrifuged (200–300 g, 10 min). Next, cell pellet was resuspended in 1 ml of BSA/PBS.

Statistical data analysis. Statistical analysis of the results was performed using MS Excel software. All data were expressed as the mean ± standard deviation, and at least three replicates were evaluated. The significance of received results between groups was evaluated by Student's *t*-test. $p < 0.05$ was considered as statistically significant.

RESULTS

Binding specificity of rabbit and mice sHB-EGF pAbs. Neutralizing anti-HB-EGF antibodies were generated by immunization of experimental animals. Firstly, we have tested the binding of pAbs to human rsHB-EGF by ELISA. Rabbit (Fig. 1, *a*) and mouse (Fig. 1, *b*) Abs showed comparable binding to the rsHB-EGF but not to the milk casein. The rabbit HB-EGF neutralizing pAbs were chosen for further research.

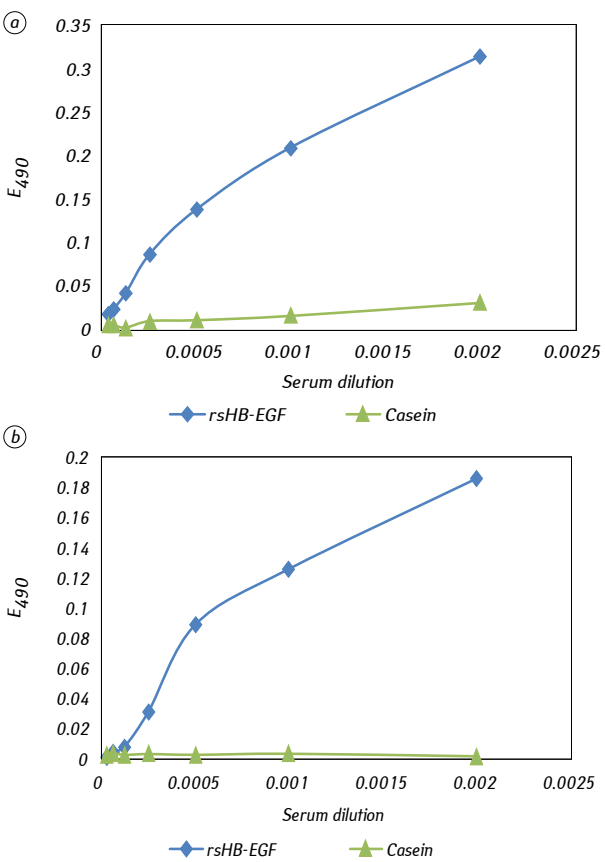

Fig. 1. IgG level to the target (rsHB-EGF) and control (casein) antigens in serum of immunized rabbits (*a*) and mice (*b*). The results of a representative experiment are presented

Then, we examined specificity of pAbs by testing their binding to human and mouse rsHB-EGF and human recombinant proHB-EGF (Fig. 2). Despite the high homology between the human and mouse HB-EGF proteins, obtained pAbs better bind with human sHB-EGF and proHB-EGF compared to mouse rsHB-EGF.

Binding specificity of pAbs to sHB-EGF on the cell surface. A431 cell line was chosen as the perfect model for investigation due to the high amount of pro-HB-EGF on the cell plasma membrane and significant EGFR overexpression. By using the method of confocal microscopy, we have shown that the HB-EGF specific pAb but not control serum binds to the surface of the A431 cells (Fig. 3).

HB-EGF neutralizing antibodies could block sHB-EGF binding with receptors on A431 cells. To investigate whether generated pAbs to human sHB-EGF could interfere with the process of receptor-ligand formation we perform flow cytometry test with A431 cells, stained with fluorescently labeled rsHB-EGF. As we can see in Fig. 4, *a*, the fluorescence peak in the histogram indicates cells incubated with mCherry-rsHB-EGF, whereas, there is no fluorescence in the case of incubation with free mCherry or unstained cells.

The addition of immune serum containing pAbs against sHB-EGF to cells results in the antigen-antibody complexes formation preventing the binding of mCherry-rsHB-EGF with cellular receptors (Fig. 4, *b,*

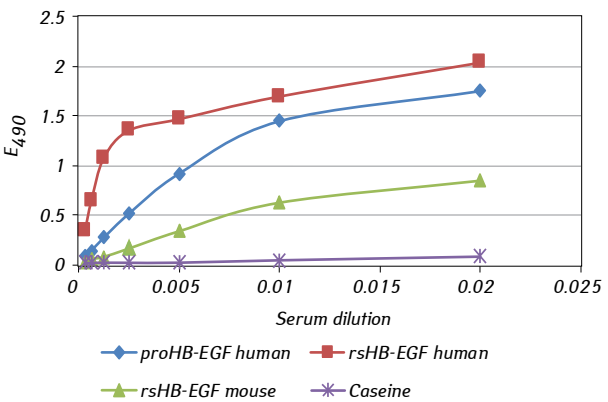

Fig. 2. The binding of rabbit anti-human sHB-EGF pAbs to various forms of human and mouse HB-EGF and control (casein) antigen. The results of a representative experiment are presented

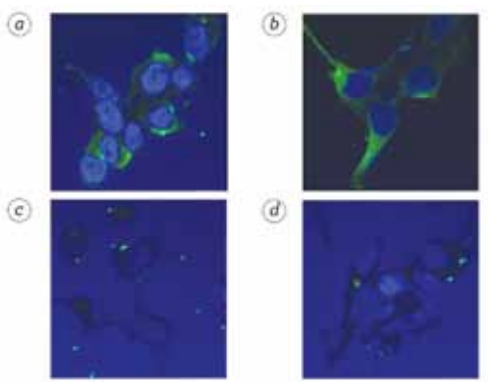

Fig. 3. Confocal images of A431 cells incubated with rabbit sHB-EGF-neutralizing pAbs (*a, b*), serum obtained before immunization (*c*) and anti-rabbit FITC conjugate only (*d*); cells nuclei stained with Hoechst 33342

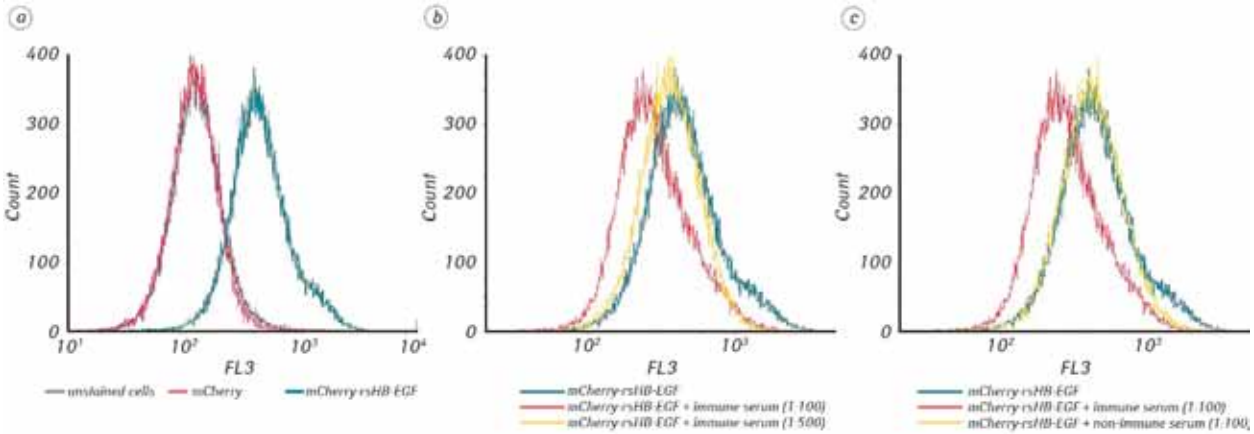

Fig. 4. Detection of the ability of pAbs to block the interaction of HB-EGF with receptors. Fluorescence intensity of A431 cells incubated with mCherry-rsHB-EGF (*a*), immune serum in different dilutions (*b*) and immune or non-immune serum (*c*)

c). It should be noted that the serum obtained from animals before immunization (non-immune serum) did not interfere with binding of mCherry-rsHB-EGF to the surface of A431 cells. Thus, pAbs obtained during the immunization block the interaction of sHB-EGF with growth factor receptors.

Neutralization of sHB-EGF mitogenic activity. We examined the effect of pAbs on the proliferation of A431 cells by comparing with non-immune serum from the same animal using MTT assay. We observed a convincing cytotoxic and antimitogenic effect of the obtained rabbit blood serum containing anti-human sHB-EGF pAbs (Fig. 5). According to the results of the MTT assay, addition of the immune serum, but not serum obtained from the same animal before immunization, inhibited metabolic and proliferative activity of A431 cells and subsequently decreased their survival. Also, the cytotoxic activity of anti-HB-EGF pAbs has taken place, provoked by the sHB-EGF blockage, which was shed from A431 cell surface in the culture medium or added to the culture medium during the investigation.

The potential effect of serum growth factors is observed when comparing the results of the MTT-test of control cells and cells after incubation with non-immune rabbit serum, which did not contain HB-EGF-neutralizing Abs (see Fig. 5). Surprisingly, intact serum significantly stimulated proliferation of A431 cells,

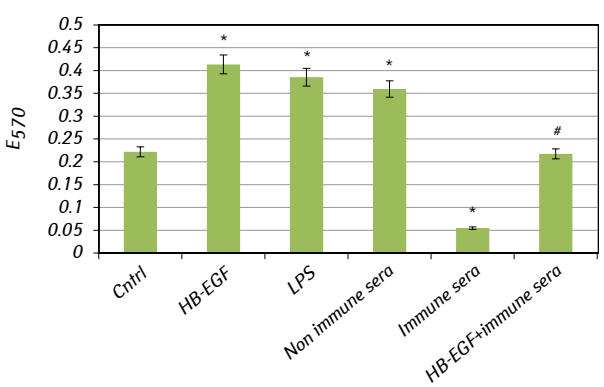

Fig. 5. Results of MTT assay of A431 cells under the influence of heat inactivated rabbit serum samples (1:100), LPS (500 ng/mL), rsHB-EGF (500 ng/mL) and equimolar mix of rsHB-EGF with anti-HB-EGF immune serum; M ± SD, n = 18, $^{*}p < 0.05$ compared to control, $^{\#}p < 0.05$ compared to sHB-EGF

apparently due to the presence of various mitogens. Besides, the cytotoxic effect of the immune serum cannot be related to the activation of the complement, since both types of serum samples have been thermally treated to inactivate the components of the complement system.

For a comparative study of the proliferative effects, exogenous rsHB-EGF, bacterial LPS that stimulates cell proliferation, and ionomycin, causing complete cell death in 24 h, were used (data not shown). The results of the proliferation test where cells were cultured in the medium containing the mix of the immune serum and rsHB-EGF did not differ from the results of the intact cells, although the presence of rsHB-EGF in the medium increased the number of cells by almost twice, and the addition of immune serum resulted in almost complete cell death.

The MTT assay data indicate the antiproliferative activity of immune serum with anti-sHB-EGF pAbs. Obtained antibodies prevent the binding of sHB-EGF with the corresponding receptors on the cell membranes and block its mitogenic effect.

Cytotoxic effect of sHB-EGF neutralizing antibodies caused by apoptotic cell death. As we show above, the blockage of sHB-EGF activity with specific pAbs lead to notable inhibition of cell survival. Next, we have assessed the cell death rate using Annexin V — EGFP — PI test. We have shown that after 24 h of incubation, in the presence of antibodies against HB-EGF in the immune serum (1:100) the number of cells actively stained with EGFP fused Annexin V increases by a quarter compared to control. However, the cells staining with PI wasn't detected, so the cellular plasmatic membrane retains the integrity and the cell dies not by the mechanism of complement-dependent cytotoxicity (Fig. 6). Thus, under the addition of anti-sHB-EGF serum in a dilution of 1:100 (but not 1:500) (see Fig. 6), a subpopulation of cells that actively binds Annexin V — EGFP increases. This process occurs due to the translocation of phosphatidylserine to the outer side of the plasma membrane, which evidence on cell apoptosis.

DISCUSSION

In clinical practice, mAbs against different growth factors receptors are commonly used as antitumor

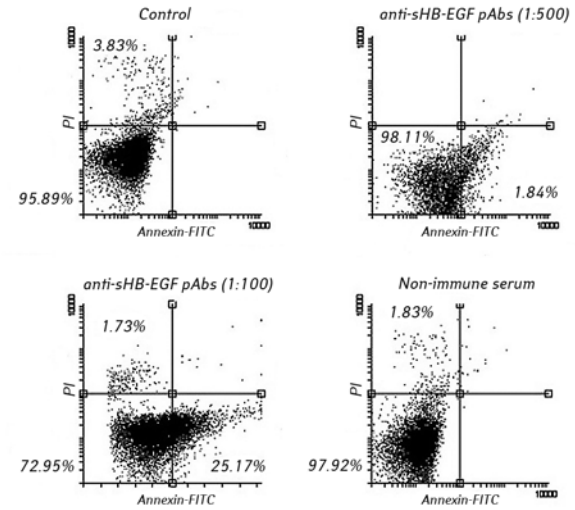

Fig. 6. Cytotoxicity assay of A431 cells with anti-HB-EGF pAbs and non-immune serum using Annexin-V-EGFP and PI labels

agents [27]. Many growth factors realize their activity through more than one receptor, so blocking one of them did not fully eliminate their effect. This is most important in the cases of cancers with elevated growth factor expression. This fact makes growth factors good targets for anticancer therapy. VEGF antibody is the first approved drug that is successfully used for the treatment of metastatic colorectal cancer [21]. HB-EGF is one of the most potent EGFR activators and its expression is increased in several cancer types [28]. So, blocking its activity may be considered promising for cancer treatment.

In this study, to assess the effect of full neutralization of sHB-EGF activity we generated and characterized pAb to human sHB-EGF. We revealed that rabbit pAb are characterized by high binding to sHB-EGF. Mouse and human HB-EGF have above 80% similarity, so human sHB-EGF was less immunogenic for mouse compared to the rabbit. Obtained rabbit pAb also demonstrated high binding to recombinant human proHB-EGF as well as binding to proHB-EGF presented on the plasma membrane of A431 cell line. Three variants of binding are possible: first, the pAbs binds with proHB-EGF, in spite of the fact that animal immunization was carried out by soluble analog rather than membrane-associated antigen; secondly, the Abs binds with HB-EGF bound with heparan sulfates proteoglycans on the cell surface; and third, the Abs binds with sHB-EGF, which is in ligand-receptor complex with the EGFR. Blocking of HB-EGF activity in A431 proliferation assay resulted in inhibition of cell growth and/or suppression of survival through apoptosis activation.

To date, several anti-HB-EGF mAbs were presented in the literature. They have different epitopes and exhibit an antiproliferative effect in various cancer cell lines. In 2010, Hamaoka et al. [19] characterized a group of mAb with epitopes in heparin-binding and EGF-like domains of HB-EGF but none of them demonstrated significant antimitogenic effect. Several obtained antibodies partly inhibited cell growth by blocking ectodomain shedding and formation of sHB-EGF. Later, another group of scientists succeeded in isolating one mAb that effectively neutralizes HB-EGF-mediated proliferation of ovarian cancer cells. This mAb has a conformational epitope and only some amino acids of HB-EGF molecule were found to be responsible for such interaction [18]. All these data leads to the idea that complete neutralization of HB-EGF activity cannot be achieved by blocking single epitope or this epitope has not been found yet.

To sum up, we used pAbs to reach maximum blocking of sHB-EGF activity and characterized their antiproliferative and apoptotic effects in A431 cells. So, searching and development of effective neutralizing agent against sHB-EGF is important and promising research direction for establishing of a new targeted antitumor therapy.

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