ISOLATION AND COMPERATIVE STUDY OF THE GROWTH INHIBITING THERMOSTABLE PROTEIN COMPLEX FROM THE BONE MARROW OF THE ADULT MICE

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The regulation of proliferation of cells with different renewal capabilities underlies homeostasis. One of the mechanisms of the regulation involves the interaction of cells with the growth factors, which are often proteins and are equally important in both the pre - and postnatal periods of development [14]. For instance, in the prenatal period of the development, the process of formation of primary germ cells is regulated by specific signaling molecules BMP [12, 13], while in the cell migration and differentiation FGF and other proteins are involved [1, 9, 22, 25]. The importance of their study was further enhanced by discovering that endogenous factors, natural products of animal cells, do not have the same toxic effects as other chemicals on the tissues and organs when exposed to transformed cells [4, 17]. The interest in these factors has increased since the discovery of the disorders in the regulation of certain growth factors in the process of wound healing both in vivo and in vitro [23]. Based on these and other similar research, the possibilities of using the growth factors, their inhibitors, and antibodies for therapeutic purposes, are still relevant.

The growth-inhibitory thermostable protein complexes (TPC) from various tissues of adult rats are isolated and partially characterized. TPC has been identified in almost all phylogenetically distant organisms - from humans to bacteria. It has been established that in adolescent animals (7-day-old rats) TPCs decrease homotypic cell proliferation by the inhibition of the transcription process [8, 10]. This complex is not characterized by species specificity, but the tissue specificity is revealed only regarding terminally differentiated cells of the adult organism. It has been shown that an adult rat's brain TPC inhibits the proliferation of progenitor cells in the Dentate Gyrus [8]. It is interesting that rat's brain TPC does not affect the proliferative activity of the mice bone marrow that is the source of blood cell renewal in the pre-and postnatal development [19,20]. It has been suggested that access to the bone marrow cells for different factors is restricted. According to the literature, the bone marrow contains actively proliferating cells, as well as the cells at different stages of differentiation. Starting with committed cells, distant mechanisms provided by various cytokines and ''keylon-antikeylon'' systems dominate in the regulation of hematopoiesis [3,24]. Based on the above mentioned, it is interesting whether the bone marrow cells contain the growth-inhibiting thermostable protein complex. The aim of the work was the identification of TPC in the bone marrow cells of adult mice and the comparative study of its action.

Material and methods. Experiments were carried out on adult (20-25 g) mice and adolescent (7 days) white rats. The animals were divided into two groups: the control group animals were injected with 100 µl 0.9% saline, and the test group animals were injected with mice bone marrow TPC (200 γ) intraperitoneally. Three hours later bone marrow and liver tissues were fixed in 4% paraformaldehyde solution prepared in 0.1 M phosphate-buffered saline pH=7.4. For the determination of the colchicine mitotic index per 1000 cells (‰) 1 mg/kg of colchicine was injected into the animals of both groups 2 hours before taking the material.

Alcoholic extraction of thermostable proteins from the adult mice bone marrow

The bone marrow of white mice was washed with saline at room temperature. Then the cold distilled water in a ratio of 1:8 was added and the tissue was homogenized in a homogenizer. The homogenate was rapidly frozen in liquid nitrogen and thawed at room temperature. To the resulting mass, the 96^0 alcohol was added to a final concentration of 50^0. The solution was then placed at + 4^0C for one hour, then centrifuged at 600g for 10 minutes on a K-23 centrifuge. 96^0 alcohol was added to the obtained supernatant in such an amount that the final concentration was 81^0, the solution was stored at + 4^0 C for one hour and centrifuged in the same manner. The obtained precipitate was dissolved in water and boiled at + 100^0 C in a water bath for 20 minutes. Then it was centrifuged, the supernatant was frozen in liquid nitrogen and lyophilized in an adsorption-condensation lyophilizer. In obtained powder, the protein was determined by the Lowry method [15].

Preparation of the material for the study under the light microscope

To examine the tissues (bone marrow, liver) under a light microscope, the materials were fixed in the 4% formaldehyde solution prepared on the Na/K phosphate buffer. After the fixation, 5% of EDTA solution was used to decalcify the femur of adolescent rats. Dehydration of the material took place in an increasing range of alcohols of different concentrations. Tissues were embedded in a wax-paraffin mixture. 5-7 µm thick slices were stained with hematoxylin-eosin. Tissue samples were studied under a light microscope (Zeiss Primo Star, Germany). To estimate the mitotic index (‰), at least 5,000 cells were counted.

Native protein electrophoresis in polyacrylamide gel

Native protein electrophoresis was performed using Davis method [5]. Acrylamide gel with a concentration gradient of 10-25% was used. The gel samples were solved in a buffer (0.5 M Tris HCl pH-6.8; 50% glycerol; 0.05% bromophenol blue) and put 20 µl of protein per sample in the gel pockets. Their electrophoretic separation was performed with power - 14 mA, voltage - 100V. At the end of the process the gel was stained with a solution of silver nitrate.

Gel staining with silver nitrate

The silver staining was performed according to the method of Nesterenko [18]. The gel was treated with a mixture of 60 ml 50% acetone and 1.5 ml 50% trichloroacetic acid for 5 minutes and then kept in distilled water for 5 minutes. After, the gel was treated with 50% acetone for 5 minutes and later with sodium thiosulfate solution (100 µl of 10% Na2S2O3 x 5H2O + 60 ml of distillate) for 1 minute. After this the gel was treated with the silver nitrate solution (0.8 mL 20% AgNO3 + 0.6 mL 37% formaldehyde + 60 mL distillate) for 8 min and then with the mixture of sodium carbonate and sodium thiosulfate (1.2 g Na2Co3 + 25 mkl 37% formaldehyde + 25 µl Na2S2O3 + 60 ml bidistilate) until staining. The reaction was stopped with a4% acetic acid solution. Between each procedure, the gel was rinsed with the distilled water 3 times for 3-5 seconds.

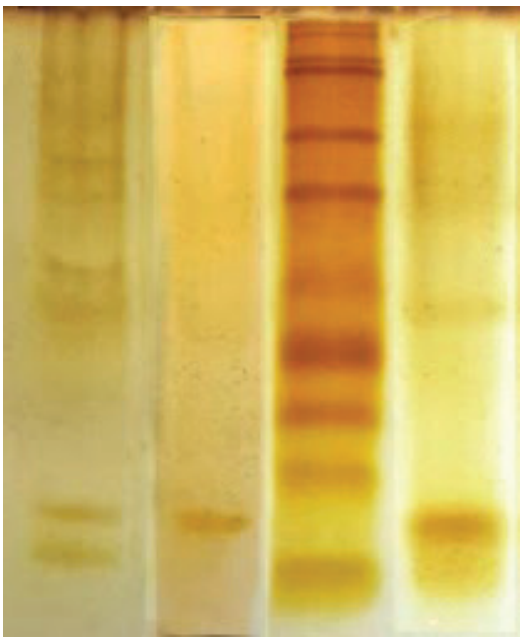
Separation of peripheral blood mononuclear cells

A density gradient of Ficoll-400 ("Sigma-Aldrich" - Sweden) was used to separate blood cells. 15 ml blood of mice treated with EDTA (to prevent clotting) was added to 10 ml Ficoll (density 1.119 g / ml) in the tube carefully to avoid mixing and centrifuged for 30 minutes at 2000 rpm. As a result of the centrifugation, there were 4 fractions in the test tube from which the suspension of mononuclear cells was taken with a pipette and transferred to a clean tube and diluted with PSB in a 1:1 ratio. Then it was centrifuged for 8 minutes at 1600 rpm. After the centrifugation 5 ml PBS was added to the precipitate, was well suspended and centrifuged for 10 min at 1000 rpm. The obtained precipitate was mixed with 1 ml PBS and counted the number of leukocytes in Goriaev's chamber [2,11].

The data are expressed as mean ± SD. Students' t-test was used for comparison among the different groups. $P<0.05$ was considered statistically significant.

Results and discussion. The method of alcoholic extraction of proteins was used in order to identify thermostable proteins in the bone marrow cells of adult mice. Our interest in bone marrow was connected to the presence of blood cells at different stages of differentiation. As it was established, in the cell culture of human T lymphoblastic leukemia, TPC did not show tissue specificity, that was explained by the presence of immature blast cells [16].

TPCs were isolated from the bone marrow and the peripheral blood cells of adult mice. To describe the complex, we used the electrophoresis method with already known pancreatic TPC and marker proteins. Fig. 1 shows the electrophorograms of this study.



1 2 3 4

Fig. 1. Native protein electrophoresis in polyacrylamide gel (10-25%), staining with silver nitrate

1 - adult mice bone marrow TPC; 2 - adult mice peripheral blood TPC; 3 - marker proteins (245-11 kD); 4 - adult rat pancreas TPC

It has been found that like the pancreatic TPC, the TPC isolated from the adult mice bone marrow and the peripheral blood both contain sub fractions of relatively high, as well as the low molecular weight. The proteins are presented in different amounts in the high molecular sub fraction as it is in the TPC isolated from other organs. It should be noted that in comparison to adult rat pancreas TPC, in low molecular weight sub fraction of mouse bone marrow TPC is characterized by a minor content, while in the case of peripheral blood TPC, the low molecular weight component is not different from pancreatic TPC (Fig. 1.). The low molecular weight fraction is the active component of the complex that has an inhibitory effect.

In the next stage of the study we investigated the effect of TPC isolated from the adult mouse bone marrow on the proliferative activity of homotypic cells (adult mouse bone marrow). The evaluation of metaphase figures in bone marrow of control and experimental group mice shows that: the mitotic index of the bone marrow cells in animals of the control group is quite high. In three hours after the injection of TPC, this activity is not changed compared with the corresponding data in control group. The obtained results show that TPC of adult mice bone marrow do not have an inhibitory effect on the proliferative activity of homotypic cells (Fig. 2).

The analysis of the literature about the growth inhibitory endogenous proteins shows that the similar protein complexes obtained from different organs of the adult organism decrease the mitotic activity of the actively proliferated homotypic, as well as heterotypic cells an average of 35-40% in adolescent animals [8,10].

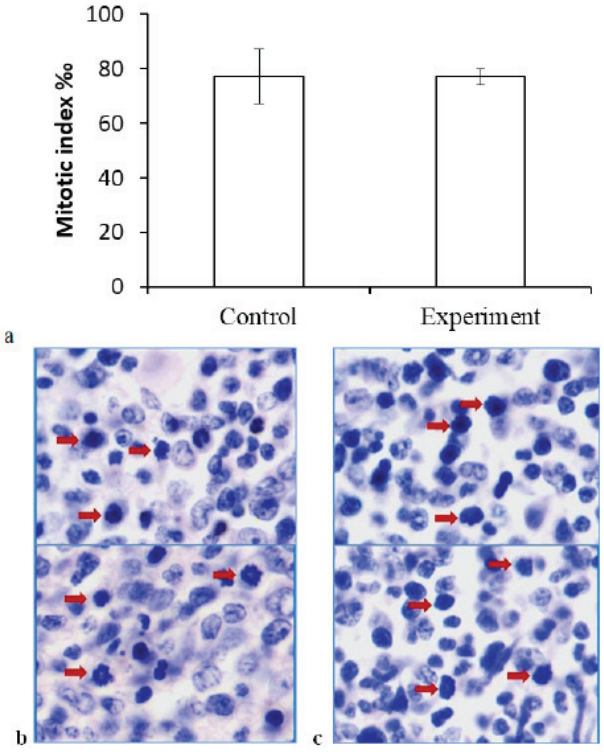


Fig. 2. The effect of the adult mice bone marrow TPC on the proliferative activity of the bone marrow of adult mice. a – mitotic index of the control and experimental group animals ($p>0,05$); b, c – the mitotic figures in control and experimental group animals bone marrow, respectively (90X7, H&E)

The question arises, how the obtained results can be explained. In spite of the fact that, the target tissue belongs to the adult organism, unlike others, it has a high proliferative activity. The access to these cells by different factors should not be restricted. The active component is presented in small quantities, in the case of above the mentioned protein complex from bone marrow that may be the reason why the effectiveness is not manifested.

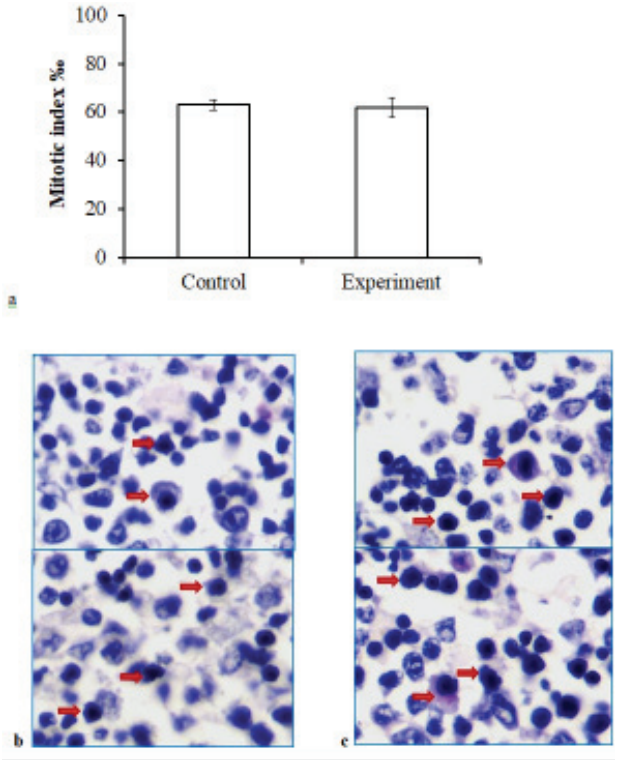


Fig. 3. The effect of adult mice bone marrow TPC on the proliferative activity of the bone marrow of 7-days old rats. a – the mitotic index of control and experimental group animals (p>0,05); b, c – the mitotic figures in control and experimental group animals bone marrow, respectively (90X7, H&E)

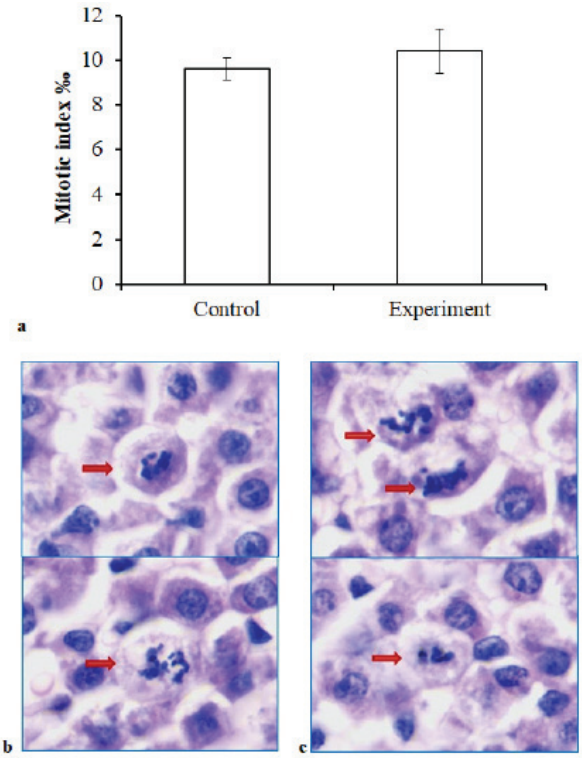


Fig. 4. The effect of adult mice bone marrow TPC on the proliferative activity of the liver tissue of 7-days old rats. a – mitotic index of control and experimental group animals (p>0,05); b, c – mitotic figures in control and experimental group animal's liver tissue, respectively (90X7, H&E)

It is known that the adult rat's TPCs isolated from different tissues are characterized by tissue specificity, that is not revealed regarding to the cells of the adolescent organism (in the early stages of postnatal development). This feature is established for the thermostable protein complex isolated from any differentiated tissue of the adult rat. Species specificity is also not typical for TPC [6,7,10].

Therefore, in the next stage of the study, we investigated the effect of mouse bone marrow TPC on the proliferative activity of adolescent rats (7 days old) bone marrow. It was found that the mitotic activity of the bone marrow in adolescent rats had not been changed after three hours of the injection of the mouse bone marrow TPC. In particular, the mitotic index of the control group was 63±2.04‰ and remained unchanged after the injection of TPC in the animals of the experimental group (Fig. 3). The results showed that the TPC isolated from the bone marrow of adult mice do not affect the homotypic cell proliferative activity of the adolescent rats.

In the next phase of the study, the effect of adult mice bone marrow TPC on the hepatocytes proliferative activity of adolescent rats (7-day-old) was studied. It was established that after three hours of the injection of bone marrow TPC, the mitotic activity of adolescent rat liver cells was not changed. In particular, the mitotic index in the control group was 9.6±0.64‰, while in the experimental group was 10.4±1.31 ‰ (Fig. 4).

Conclusions. The results of our study show that the endogenous protein complex isolated from the bone marrow cells differs significantly from all other protein complexes obtained from intact tissues. It differs in the quantitative content of components as well as in affect ability. According to the obtained data, the inhibitory effect of the mentioned complex is not manifested on the proliferation of either homotypic or heterotypic tissue cells.

The second important difference is the minor content of active component in the bone marrow protein complex. Such minor content is described in the case of the protein complex derived from benign vascular tumor (children hemangioma) cells. It has been shown that this complex does not inhibit the mitotic activity of cells in the proliferative tissues of adolescent rats [21]. It should be noted that the bone marrow is dynamically renewable hematopoietic tissue and contains several types of cell populations, the first two of which are pluripotent and multipotent stem cells. They differ by the expression of membrane receptors. However, the nature of the regulatory factors and their accessibility to cells are different [3].

In view of all the above, the fact that the bone marrow protein complex does not affect the proliferative activity of homotypic cells is explained by the structural and functional characteristics of the bone marrow. In the case of heterotypic cells, one of the reasons is the minor content of the active component (low molecular weight fraction) in the complex.

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SUMMARY

ISOLATION AND COMPERATIVE STUDY OF THE GROWTH INHIBITING THERMOSTABLE PROTEIN COMPLEX FROM THE BONE MARROW OF THE ADULT MICE

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The growth inhibiting thermostable protein complex (TPC) from the bone marrow cells of adult mice was isolated and partially characterized. A comparative analysis of TPC components was performed by polyacrylamide gel electrophoresis. It has been established that the complex isolated from the bone marrow of adult mice, like complexes obtained from other organs, contains two relatively high-molecular-weight and low-molecular-weight sub fractions of proteins. In addition, minor content of low molecular weight components was detected in bone marrow TPC. It has been established that the thermostable protein complex of adult mice bone marrow cells does not have the ability to inhibit the proliferation of homotypic cells. This may be due to the minor content of active component (low molecular weight subfraction) in the complex and the structural and functional properties of constantly renewable hematopoietic tissue, such as the nature of factors that regulate proliferation, their different cell penetration and the expression of membrane receptors.

Keywords: White mouse, bone marrow, thermostable protein complex, mitotic index.

РЕЗЮМЕ

ВЫДЕЛЕНИЕ И СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ КОМПЛЕКСА ТЕРМОСТАБИЛЬНЫХ БЕЛКОВ ИЗ КОСТНОГО МОЗГА ВЗРОСЛЫХ МЫШЕЙ

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Из клеток костного мозга взрослых мышей изолирован и частично охарактеризирован рост-ингибирующий термостабильный белковый комплекс (ТБК). Для сравнительного анализа компонентов ТБК применялся метод электрофореза в полиакриламидном геле.

Установлено, что белковый комплекс, выделенный из костного мозга взрослых мышей, как и комплексы, ранее полученные из других органов, содержит две субфракции белковых компонентов - относительно высокомолекулярную и низкомолекулярную. При этом, в ТБК костного мозга обнаружено незначительное содержание низкомолекулярных компонентов.

Установлено, что ТБК клеток костного мозга взрослых мышей не обладает способностью подавлять пролиферацию гомотипических клеток, что, по всей вероятности, обусловлено незначительным содержанием в комплексе ингибирующего рост активного начала (низкомолекулярная субфракция), а также структурными и функциональными свойствами постоянно возобновляемой кроветворной ткани: свойство факторов, регулирующих пролиферацию, экспрессия мембранных рецепторов и их афинность к действующим факторам.

რეზიუმე

ზრდასრული თაგვების ძვლის ტვინიდან ზრდის შემაკავებელი თერმოსტაბილური ცილების კომპლექსის გამოყოფა და შედარებითი შესწავლა

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ზრდასრული თაგვების ძვლის ტვინის უჯრედებიდან გამოყოფილია და ნაწილობრივ დახასიათებულია ზრდის შემაკავებელი თერმოსტაბილური ცილების კომპლექსი (თცკ). ჩატარებულია თცკ-ს კომპონენტების შედარებითი ანალიზი პოლიაკრილამიდის გელში ელექტროფორეზის გზით. დადგენილია, რომ ზრდასრული თაგვების ძვლის ტვინიდან გამოყოფილი ცილოვანი კომპლექსი, სხვა ორგანოებიდან მიღებული კომპლექსების მსგავსად, შეიცავს ცილოვანი კომპონენტების ორ, შედარებით მაღალმოლეკულურ და დაბალმოლეკულურ ქვეფრაქციებს. ამასთან, ძვლის ტვინის თცკ-ში გამოვლინდა დაბალმოლეკულური კომპონენტის მინორული შემცველობა. დადგენილია, რომ ზრდასრული თაგვების ძვლის ტვინის უჯრედების თერმოსტაბილური ცილების კომპლექსს არ გააჩნია ჰომოტიპური უჯრედების გამრავლებაზე დამთრგუნველი ზემოქმედების უნარი. ეს შეიძლება განპირობებული იყოს კომპლექსში აქტიური საწყისის (დაბალმოლეკულური ქვეფრაქცია) მინორული შემცველობით და მუდმივად განახლებადი სისხლმბადი ქსოვილის სტრუქტურული და ფუნქციური თავისებურებებით, როგორებიცაა: პროლიფერაციის მარეგულირებელი ფაქტორების ბუნება, მემბრანული რეცეპტორების ექსპრესია და მათი უჯრედებისადმი განსხვავებული წვდომა.

LASER INDUCED FLUORESCENCE OF SKIN: SUPERPOSITION OF SPECTRAL INTENSITIES

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Optical methods are widely used in biology and medicine as they allow to study objects in real-time. In particular, in those cases, the diagnosis is made by an effective non-invasive method. A large number of works are devoted to the determination and monitoring of the state of body health by determining the tissues states by their optical parameters (see for example [1-