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ABILITY OF THYMIC MSCs AND THEIR DERIVATIVES TO INTERACT WITH THE CELLS OF LYMPHOID ORIGIN

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The aim of the research was to determine the ability of thymic multipotent stromal cells and their derivatives to interact with lymphocytes obtained from different sources. It was shown that a part of thymic cells from 6–8-weeks-old C57BL mice *in vitro* were characterized by such properties: the ability to adhere to the surfaces of cell-culture plastic, the specific fibroblast-like morphology, and the ability to directed adipogenic and osteogenic differentiation. Due to these properties, the cell populations isolated from thymus could be attributed to the multipotent mesenchymal stromal cells (MMSCs) or mesenchymal stem cells (MSCs). We have shown that all types of stromal cells have an ability to interact with the lymphoid cells obtained from different sources (thymocytes, splenocytes, cells of the lymph nodes and bone marrow). The largest number of intercellular associations has been formed with the thymocytes, and the smallest one — with the lymphoid cells of bone marrow. Among differentiated forms osteogenic cells are capable to create higher number of intercellular associations, as compared to adipocytes. Thus, probably the intercellular contact interactions between the MSCs and hematopoietic cells might be used as one of the new approaches for efficient and directed modification of the cell properties.

Key words: mesenchymal stem cells from thymus, MSCs, differentiation, lymphoid cells, intercellular contacts.

A population of multipotent stromal cells reside within the bone marrow and other adult tissues such as: human umbilical cord, amniotic membrane, adipose tissue [1], placenta [2], fetal liver [3]and thymus [4]. These cells are frequently referred to as multipotent mesenchymal stromal cells (MMSCs) or mesenchymal stem cells (MSCs), which are able to differentiate into different skeletal tissues such as bone, cartilage and fat. MSCs offer significant therapeutic potential, particularly in orthopaedic applications, but may also play broader roles in regenerative medicine and cancer treatment, as anti-inflammatory, immunosuppressive agents and vehicles for gene/protein therapy. The development of new approaches to cell transplantation is currently one of the main purposes of cellular biology and biotechnology. Much attention has been focused on understanding MSCs biology. MSCs of different origin may have different therapeutic benefits.

In 1978 R. Schofield proposed a hypothesis of hematopoietic stem cells (HSCs) niche (specialized cell microenvironment). An existence of osteoblastic and vascular niches for HSCs has been postulated since 2003. One of the main components within vascular niche composition is the MSCs, the perivascular progenitors and endothelial cells, in the endosteal niches — cells of the osteogenic lineage. The MSCs may actively influence on the HSCs both via direct and humoral contacts [5].

The thymus is a vascularized organ that plays a pivotal role in T-lymphocyte development, and its durability in providing a robust acquired immune response throughout the lifespan may be partially attributed to its ability to regenerate after injury, infection, or partial surgical resection. It is postulated that there are thymic niches exist in which, with an involvement of MSCs, the conditions are created for differentiation of the T-cell progenitors migrating to the thymus from the

bone marrow [6, 7]. Thymic mesenchyme is necessary for its embryonic morphogenesis and plays a direct role in the lymphopoiesis and cell migration inside the thymus [8, 9] .

Clearly, the interactions between MSCs and HSCs play a key role in the tissues of the central organs of the immune system. However, the MSCs are also important due to their interactions with the hematopoietic cells on the periphery. They inhibit the post-transplant reactions and stimulate the productive phase of antibody synthesis [10] as well as suppress the processes associated with the proliferation of T-lymphocytes in the different in vitro systems [11]. Thus the MSCs influence not only on the HSCs but also on the various populations and subpopulations of the lymphocytes [12–14]. That is, the interaction between MSCs and other cells in the organism is necessary and perhaps it occurs both in the physiological conditions and during the pathological processes.

In our previous studies it was found that the co-cultivation of thymic MSCs with thymocytes potentiates the osteogenic differentiation of the former. It is not excluded that thymocytes produce certain cytokines which also contribute to the change of the MSCs properties to enhance their differentiation capacity. But in the experimental conditions when the MSCs and thymocytes are in a direct contact during 24 hours, the cell-cell interaction can play a certain role in the potentiation of the osteogenic differentiation [15].

Taking into account the above-mentioned, we decided to study the ability of MSCs and their differentiated derivatives to the cell-cell interaction with the lymphoid cells obtained from different sources (thymocytes, splenocytes and cells of the lymph nodes and bone marrow).

Materials and Methods

6–8 weeks-old male C57BL mice were used in our experiments. Animals were received from the vivarium of the Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAMS of Ukraine. Mice had *ad libitum* access to balanced food and water. All the experiments were conducted according to the article 26 of the Law of Ukraine "On protection of animals from cruelty" dated 21.02.2006 and the European Convention for the Protection of the Vertebrate Animals used for the Experimental and other Scientific Purposes (Strasburg, 1986).

Mice were euthanized by cervical dislocation, the thymuses were isolated in sterile conditions. The culturing of thymic stromal cells was performed using standard explants technique [16] in DMEM/F12 1:1 nutrient mixture (Sigma, USA) with addition of 10% fetal bovine serum (Sigma, USA), 10 мМ L-glutamine (Sigma, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Darnytsia, Ukraine), in the CO_2-incubator (Jouan, France) at 37 °C and 5% CO_2. One basic requirement when defining MSCs was the attachment of these cells to plastic surfaces. The adherence of our MSCs to plastic surfaces and characteristic fibroblast-like morphology were actually the visible selection markers. Cells were subcultured in the proportion 1:3 using the 0.05 % trypsin (Biotestmed, Ukraine) and 0.02 % EDTA (Sigma, USA).

The thymocytes, splenocytes and cells of the lymph nodes were obtained by the preparation of the organ with the needles, and bone marrow suspensions were obtained by the flushing of femoral bones with nutrient mixture. Additionally, the procedure included a 30 min incubation of the isolated suspension in dishes with untreated silicone surface at 37 °C to remove the adherent cells.

To obtain differentiated forms MSCs were cultured in appropriate differentiation media. The osteogenic differentiation was induced in DMEM/F12 1:1 nutrient mixture (Sigma, USA) supplemented with 15% fetal bovine serum (Sigma, USA), containing 10 mmol L-glutamine (Sigma, USA), 50 μg/ml L-ascorbic acid (Sigma, USA), 10 mmol β-glycerophosphate (Sigma, USA), 0.1 μmol dexamethasone (Sigma, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Darnytsia, Ukraine). The adipogenic differentiation was induced in DMEM high glucose (4.5 g/L) medium (PAA, Germany) supplemented with 10% horse serum (PAA, Germany), containing 10 mmol L-glutamine (Sigma, USA), 0.5 μmol dexamethasone (Sigma, USA), 6 μg/ml insulin (Sigma, USA) and 100 U/ml penicillin and 100 μg/ml streptomycin (Darnytsia, Ukraine). Cultivation was performed in the CO_2-incubator (Jouan, France) at 37 °C and 5% CO_2 for 10 days. The effectiveness of osteogenic and adipogenic differentiation was assessed by the staining with 1% solution of alizarin red S (Sigma, USA) and with 0.2% solution of oil red O (Sigma, USA), respectively [16]. Alizarin red and oil red were extracted by 10% solution of the acetic acid or isopropyl alcohol, respectively. Intensity of staining of the obtained extracts

was estimated in 96-well plate by optical density measuring at 520 nm using spectrophotometer Sunrise (Tecan, Austria).

To determine the ability to interact of the cell suspensions with a mixture of MSCs or their differentiated forms (10^5/ml, 50 µl) and lymphocytes (10^7/ml, 50 µl) obtained from different lymphoid organs, cells were centrifuged at 250 g, the precipitate was then resuspended and microscopically examined. Calculations unit fibroblast-like cells and those that have attached three or more lymphoid cells were conducted.

The obtained results were analyzed statistically using MS Office Excel software (Microsoft, USA), using the Shovene criterion for abnormality of values [17].

Results and Discussion

The thymic stromal cells that were cultured in the osteogenic or adipogenic induction medium, were well-stained with the alizarin red and oil red solution respectively, in contrast to the control cells grown in standard medium without the addition of inductors, that confirm effectiveness of directed osteogenic and adipogenic differentiation of thymic MSCs (Fig. 1, 2).

Following osteogenic induction, large amounts of mineralized extracellular matrix were formed. Adipogenic induction led to extensive intracellular lipid droplet accumulation, while control cultures showed no lipid droplets.

International Society for Cellular Therapy (ISCT) defined multipotent MSCs by their adherence to cell-culture plastic, specific surface antigen expression pattern, and their multipotent mesenchymal differentiation potential [18]. The most important confirmation is the capability to differentiate into the osteogenic, chondrogenic, and adipogenic lineages *in vitro* which is one of the most fundamental characteristics of MSCs [19].

Just in the last few years evidence has appeared that the neonatal thymus contains MSCs fulfilling the minimal criteria postulated by the ISCT and differentiating into all of the characteristic mesenchymal cell lineages [20].

In general, it is shown by us that thymic MSCs from 6–8 weeks-old mice alike MSCs from other origins are able to differentiate

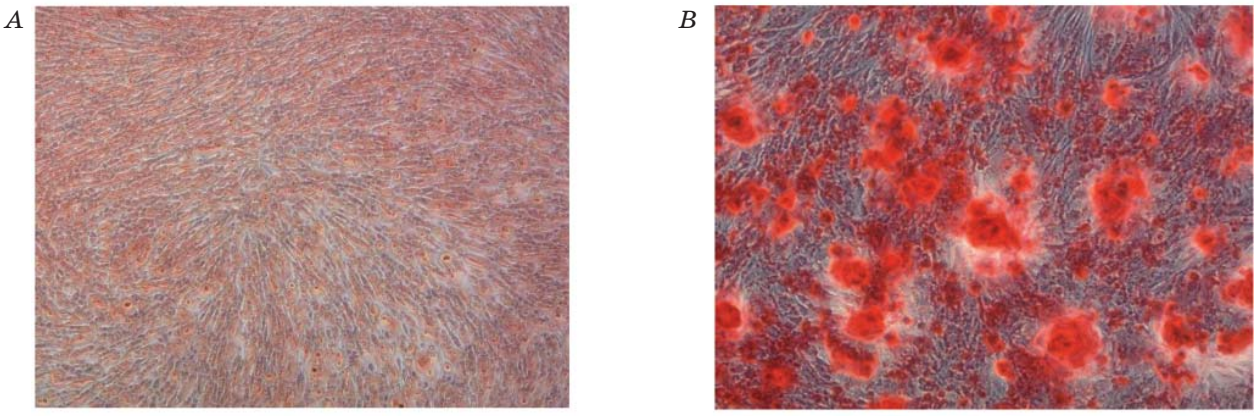

Fig. 1. **Microphotographs of the thymic MSCs cultures, 10^{th} day of cultivation in: standard (*A*) or osteogenic (*B*) culture medium.** Alizarin red staining. Oc. ×10, ob. ×10

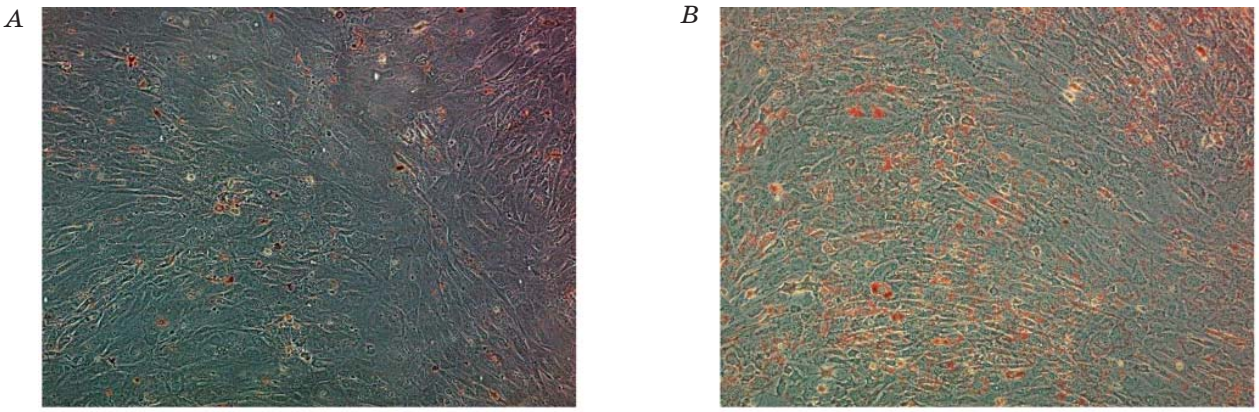

Fig. 2. **Microphotographs of the thymic MSCs cultures, 10^{th} day of cultivation in: standard (*A*) or adipogenic (*B*) culture medium.** Oil red O staining. Oc.×10, ob. ×10

in vitro into adipocytes and osteoblasts. This data together with the adherence to plastic surfaces under standard culture conditions and fibroblast-like morphology indicate that isolated cells have characteristics of MSCs.

It was known that thymic MSCs have ability to interact with hematopoietic cells [21], but there was no information about this ability of their differentiated derivatives.

Our data shows that MSCs and all studied types of differentiated stromal cells (osteoblasts, adipocytes) and lymphoid cells obtained from different sources (thymocytes, splenocytes, cells of the lymph nodes and bone marrow) are able to interact with each other (Fig. 3). The intercellular contacts were characterized by the formation of associations between the fibroblast-like cells and the lymphoid cells. MSCs, osteoblasts and especially thymocytes, exhibit significantly greater ability to form intercellular associations than other types of cells.

Thymocytes, splenocytes, and lymph node cells create approximately the same number of associations with all three types of stromal cells. But there is a trend to higher activity of thymocytes in these interactions.

A significantly lower number of associations is formed the MSCs and adipocytes with lymphoid cells of bone marrow. However, there is a significantly higher number of associations between osteoblasts and lymphoid cells of bone marrow, in contrast to splenocytes and cells of the lymph nodes.

The opposite observation concerns a different activity of thymocytes and lymphoid cells of bone marrow. In previous studies, it has been shown that these types of cells have a different effect on the differentiation potential of MSCs. The co-cultivation of MSCs with thymocytes potentiated osteogenic differentiation [15]. Probably the intensive intercellular contact that we have demonstrated in this study plays a role in the influence of thymocytes on the functional activity of thymic MSCs.

It should be noted that activation of the MSCs and hematopoietic cells after contact may have great importance in the pathology progress, when a disbalance and dysfunctions

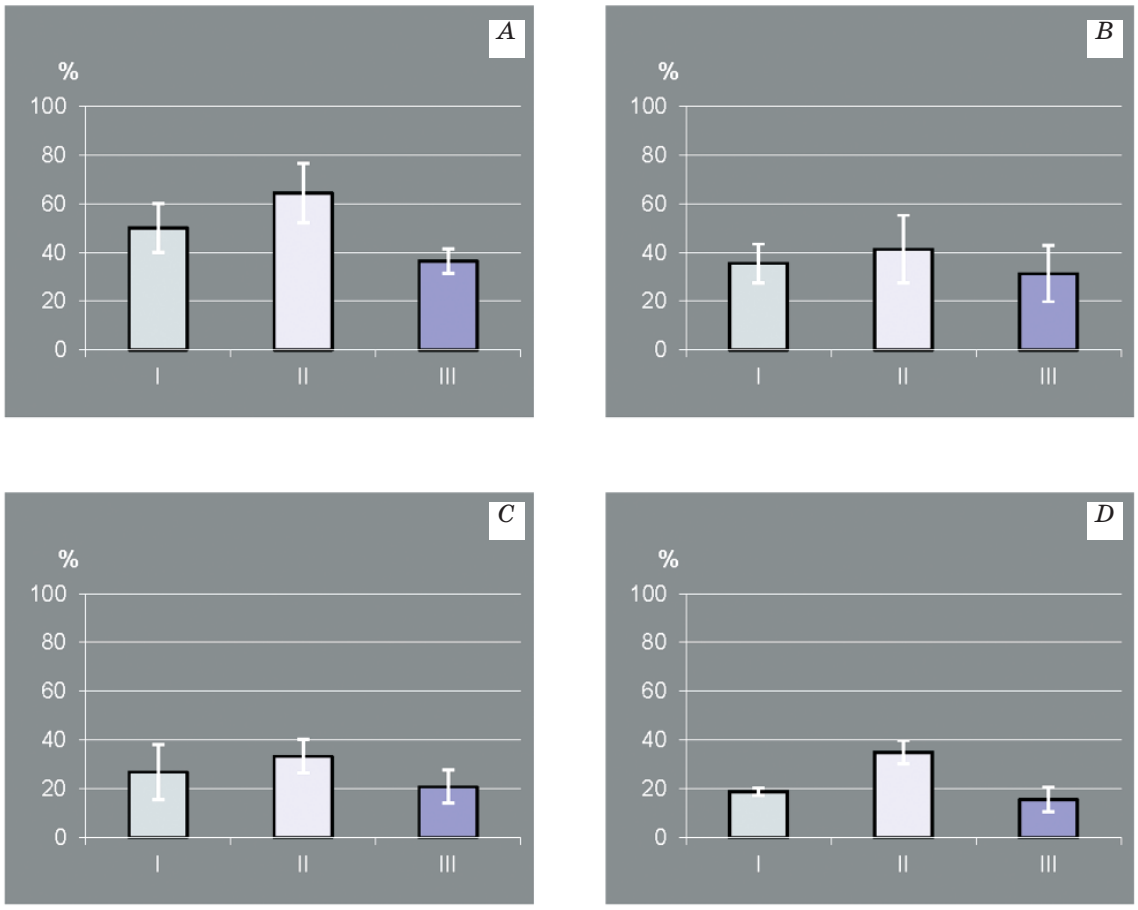

Fig. 3. **Percentage of multipotent stromal cells:**
of thymus (I), osteoblasts (II) and adipocytes (III), coupled with several thymocytes (*A*), splenocytes (*B*), cells of the lymph nodes (*C*) and bone marrow (*D*) with the formation of cellular associations.
* — $P < 0.05$ — the difference is significant compared to the osteoblasts

of the stromal cells can be mediated by the quantitative and functional disruption in the subpopulations of the immune cells. Further investigations in this direction will promote progress in our knowledge about the systemic and local pathology. On the other hand, contact interaction between MSCs and hematopoietic cells might be used as one of the new approaches for efficient and directed change in the properties of cell transplants, which leads to significant activation of the interacting cells.

Thus, we have been demonstrated a tendency to different ability of thymic MSCs and their derivatives to contact interacts with different lymphoid cells. The largest number of intercellular associations are formed with thymocytes and the lowest one – with the lymphoid cells of bone marrow. Among differentiated forms osteogenic cells created higher number of associations as compared to adipocytes.

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ЗДАТНІСТЬ МСК ТИМУСУ ТА ЇХНІХ ПОХІДНИХ ДО ВЗАЄМОДІЇ З КЛІТИНАМИ ЛІМФОЇДНОГО ПОХОДЖЕННЯ

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Метою дослідження було визначити здатність мультипотентних стромальних клітин тимусу та їхніх похідних до взаємодії з лімфоцитами, отриманими із різних джерел. Показано, що клітини тимусу 6–8-тижневих мишей C57BL у культурі характеризуються такими властивостями: спроможністю адгезувати до поверхонь культурального пластикового посуду, специфічною фібробластоподібною морфологією та здатністю до спрямованого адипогенного й остеогенного диференціювання. За цими властивостями клітинні популяції, виділені з тимусу, можна віднести до мультипотентних мезенхімальних стромальних клітин (ММСК) або мезенхімальних стовбурових клітин (МСК). Ми показали, що всі типи стромальних клітин можуть взаємодіяти з лімфоїдними клітинами, отриманими із різних джерел (тимоцити, спленоцити, клітини лімфатичних вузлів та кісткового мозку). Найбільшу кількість міжклітинних асоціацій вони утворювали з тимоцитами, найменшу — з лімфоїдними клітинами кісткового мозку. Серед диференційованих форм остеогенні клітини утворювали більше міжклітинних асоціацій порівняно з адипоцитами. Таким чином, міжклітинні контактні взаємодії між МСК та гемопоетичними клітинами, ймовірно, можуть бути використані як один із нових підходів до ефективної та спрямованої зміни властивостей клітин.

Ключові слова: мезенхімальні стовбурові клітини тимусу, МСК, диференціація, лімфоїдні клітини, міжклітинні контакти.

СПОСОБНОСТЬ МСК ТИМУСА И ИХ ПРОИЗВОДНЫХ К ВЗАИМОДЕЙСТВИЮ С КЛЕТКАМИ ЛИМФОИДНОГО ПРОИСХОЖДЕНИЯ

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Целью исследования было определить способность мультипотентных стромальных клеток тимуса и их производных к взаимодействию с лимфоцитами, полученными из различных источников. Показано, что клетки тимуса 6–8-недельных мышей C57BL в культуре характеризуются такими свойствами: способностью адгезировать к поверхностям культуральной пластиковой посуды, специфической фибробластоподобной морфологией и способностью к направленной адипогенной и остеогенной дифференцировке. По этим свойствам клеточные популяции, выделенные из тимуса, можно отнести к мультипотентным мезенхимальным стромальным клеткам (ММСК) или мезенхимальным стволовым клеткам (МСК). Все типы стромальных клеток могут взаимодействовать с лимфоидными клетками, полученными из различных источников (тимоциты, спленоциты, клетки лимфатических узлов и костного мозга). Наибольшее количество межклеточных ассоциаций они образуют с тимоцитами, наименьшее — с лимфоидными клетками костного мозга. Среди дифференцированных форм остеогенные клетки образуют больше межклеточных ассоциаций по сравнению с адипоцитами. Таким образом, межклеточные контактные взаимодействия между МСК и гемопоэтическими клетками, по-видимому, могут быть использованы как один из новых подходов к эффективному и направленному изменению свойств клеток.

Ключевые слова: мезенхимальные стволовые клетки тимуса, МСК, дифференцировка, лимфоидные клетки, межклеточные контакты.