Cell and Organ Transplantology. 2016; 4(2):220-223.
doi:10.22494/cot.v4i2.59

Potentiation of directed osteogenic differentiation of thymic multipotent stromal cells by prior co-cultivation with thymocytes

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ABSTRACT

It is known that multipotent stromal cells (MSCs) and thymocytes possess membrane affinity and interaction in the thymic niches that is essentially important for thymocytes differentiation. However there are no data about possible influence of intercellular contacts in the reverse direction: from the thymocytes to the MSCs.

MATERIALS AND METHODS. *The MSCs were obtained from the thymuses of C57BL mice, using the explants technique, and cultivated under standard conditions during 8-12 passages. Thymocytes or bone marrow cells (10^6) were added to $4 \cdot 10^4$ MSCs for 24 hours. Thereafter they were eliminated and standard culture medium was changed by osteogenic or adipogenic differentiation medium and cultured during 10 days. After fixation the cells were stained by 1 % alizarin red S solution or 0.2 % solution of oil red O respectively. After extraction of the stains with 10 % acetic acid or isopropyl alcohol the optic density of extracts at 520 nm was measured.*

RESULTS. *We found that thymic multipotent stromal cells of the C57BL mice were effectively differentiated in vitro into the osteogenic and adipogenic lineages in the appropriate differentiation media that was evidenced by alizarin red and oil red staining of cell cultures. According to the results of measurement of optic density of the dye extracts, it was found that effectiveness of thymic MSCs differentiation into the osteogenic lineage after prior short-term co-cultivation with the thymocytes is increased.*

CONCLUSIONS. *The contact of thymic stromal cells with thymocytes but not with bone marrow cells in the previous 24 hours potentiates the osteogenic differentiation and has no effect on the adipogenic cells maturation.*

KEYWORDS: *thymic multipotent stromal cells; thymocyte, bone marrow cells; osteogenic differentiation; adipogenic differentiation*

Interaction of the multipotent stromal cells (MSCs) with hematopoietic cells has been intensively studied. In the 1978 year the hypothesis was formulated about the existence of a microenvironment for the hematopoietic stem cells (HSCs) in the bone marrow (BM), which is composed of various cell and tissue components forming the niches [1]. Today the presence of such functional units is beyond question. There are the endosteal and vascular niches within the bone marrow in which, owing to the complex intercellular interactions, adhesion molecules, cytokines and extracellular matrix, the HSCs maintained at dormant state their self-maintenance and realization of multipotency. One of the main components within vascular niche composition is the MSCs, their 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 [2]. It is postulated that there exist the thymic niches 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 [3]. Thymic mesenchyme is necessary for its embryonic morphogenesis and plays a direct role in the lymphopoiesis and cell migration in the thymus [4].

Clearly, the interaction between MSCs and HSCs plays a key role in the tissues of the central organs of the immune system. However, the MSCs are also important during their interaction with the hematopoietic cells on the periphery. They suppress the post-transplant reactions and stimulate the productive phase of antibodies synthesis [5] as well as suppress the processes associated with the proliferation of T-lymphocytes in the different *in vitro* systems [6]. Thus the MSCs influence not only on the HSCs but also on the various populations and subpopulations of the lymphocytes [7-10]. That is, the MSCs-HSCs interaction in the organism is necessary and, perhaps it occurs both, in the physiological conditions and during the pathological processes.

There appears a question: is the effect within the pair MSCs-HSCs focused in the direction of the hematopoietic cells or if this influence extends on the stromal cells too? The main property of the MSCs is ability to differentiate into multiple lineages. It can be assumed that interaction with the hematopoietic cells primarily influence upon these processes. There are few data about the influence of the hematopoietic cells on the MSCs differentiation. The data have been obtained showing that non-adhesive cells of BM as well as the LSK cells (Lin-Sca-1$^+$c-kit$^+$) co-cultured with the bone marrow MSCs and separated by the millipore membrane improve the MSCs proliferation and differentiation being registered by the increasing number of the colony-forming units of the fibroblasts and osteoblasts. The effect was related to the action of cytokines and growth factors (IL-6, SDF-1, osteocalcin), the secretion of which was significantly enhanced by the osteoblasts in the presence of the hematopoietic cells [11]. There are a few publications showing that the thymic MSCs cells are capable for multi-lineage differentiation [12], though the influence of the hematopoietic cells on this process has not been studied.

Taking into account above-mentioned, we have chosen to study the possibilities of thymic MSCs differentiation into osteogenic and adipogenic direction *in vitro* and to estimate effects on these processes of the contact interaction between the MSCs and thymocytes or bone marrow cells.

MATERIAL AND METHODS

In our experiments 6-8 weeks-old male C57BL mice were used received from the vivarium of the R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NMS of Ukraine. The animals had a free access to balanced food and water ad libitum. 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).

After mice euthanasia by cervical dislocation under ether narcosis the thymus and femoral bones were prepare in sterile conditions. The culture of thymic stromal cells was performed using standard explants technique [13] 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. Cells were subculture in the proportion 1:3 using the 0.05 % trypsin (*Biotestmed*, Ukraine) and 0.02 % EDTA (*Sigma*, USA). The thymocytes for co-culture were obtained by preparation the thymus with the needles, and bone marrow suspensions obtained by the flushing of femoral bones with nutrient mixture. Additionally, the procedure included a 30 min keeping of the isolated suspension in dishes with untreated silicone surface at 37 °C to remove the adherent cells.

Thymic MSCs of 8-12 passages with no signs of senescence were seeded into the 24-well plate ($4 \cdot 10^4$ cells per well). Once the cell monolayer was formed in the course of 24 hours, 10^6 thymocytes or bone marrow cells were added into each well and then removed the next day by washing. The thymic MSCs were cultured further in standard medium for 24 hours and then the medium was replaced with the osteogenic or adipogenic for directed differentiation in the respective wells.

The osteogenic differentiation was induced in DMEM/F12 1:1 nutrient mixture (*Sigma*, USA) supplemented with 15 % fetal bovine serum (*Sigma*, USA), containing 10 мМ L-glutamine (*Sigma*, USA), 50 µg/mL L-ascorbic acid (*Sigma*, USA), 10 мМ β-glycerophosphate (*Sigma*, USA), 0.1 µM 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 мМ L-glutamine (*Sigma*, USA), 0.5 µM 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 during 10 days.

The effectiveness of differentiation was assessed by staining with 1 % solution of alizarin red S (*Sigma*, USA) for osteogenic lineage and with 0.2 % solution of oil red O (*Sigma*, USA) for adipogenic [13]. 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).

Statistically, the obtained results were analyzed using MS Office Excel software (*Microsoft*, USA), using the Shovene criterion for abnormality of values and Student's t-test for comparison of the differences between the groups [14].

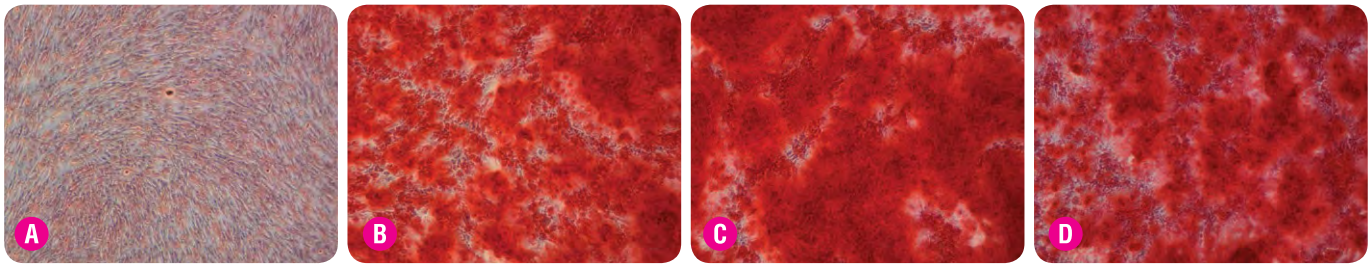

Fig. 1. Microphotographs of the thymic MSCs cultures, 10th day of cultivation in standard (A) or osteogenic (B, C, D) culture medium and after a prior 24-hour contact with the thymocytes (C) or bone marrow cells (D). Alizarin red staining. Oc. x10, ob. x10.

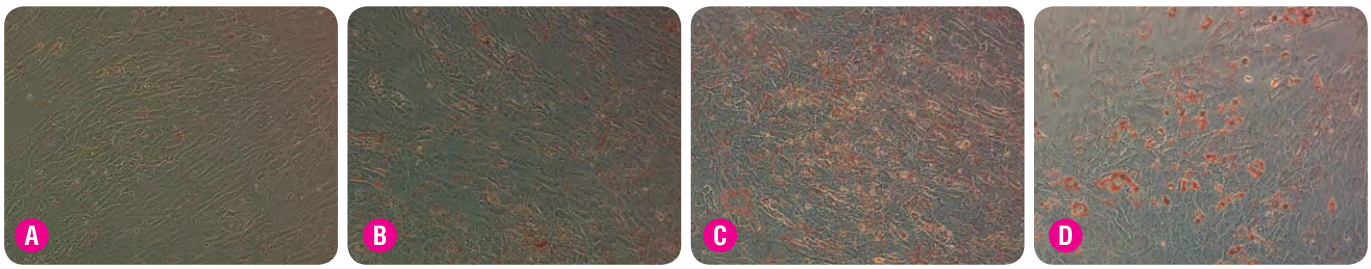

Fig. 2. Microphotographs of the thymic MSCs cultures, 10th day of cultivation in standard (A) or adipogenic (B, C, D) medium and after a prior 24-hour contact with the thymocytes (C) or bone marrow cells (D). Oil red staining. Oc. x10, ob. x10.

STATISTICS	THYMIC MSCs CULTURES			
	STANDARD GROWTH MEDIUM	OSTEOGENIC GROWTH MEDIUM		
	-	-	PRIOR CO-CULTIVATION WITH THYMOCYTES	PRIOR CO-CULTIVATION WITH BM CELLS
M ± m, units	0.022 ± 0.003	0.344 ± 0.013	0.391 ± 0.019	0.349 ± 0.008
n	5	6	6	5
min	0.018	0.299	0.335	0.331
max	0.034	0.384	0.463	0.378
$p(t)_1$	-	< 0.05	< 0.05	< 0.05
$p(t)_2$		-	< 0.05	> 0.05
$p(t)_3$			-	< 0.05

Table 1. The optical density of alizarin red S extracts after staining of the thymic stromal cells culture

Notes:
$p(t)_1$ – compared with cells grown in the standard medium without addition of differentiation factors;
$p(t)_2$ – compared with cells grown in differentiation medium;
$p(t)_3$ – compared with cells grown in differentiation medium after prior co-cultivation with thymocytes.

STATISTICS	THYMIC MSCs CULTURES			
	STANDARD GROWTH MEDIUM	ADIPOGENIC GROWTH MEDIUM		
	-	-	PRIOR CO-CULTIVATION WITH THYMOCYTES	PRIOR CO-CULTIVATION WITH BM CELLS
M ± m, units	0.196 ± 0.003	0.315 ± 0.013	0.286 ± 0.003	0.377 ± 0.010
n	5	6	5	6
min	0.190	0.267	0.278	0.346
max	0.208	0.356	0.297	0.414
$p(t)_1$	-	< 0.05	< 0.05	< 0.05
$p(t)_2$		-	> 0.05	< 0.05
$p(t)_3$			-	< 0.05

Table 2. The optical density of oil red O extracts after staining of the thymic stromal cells culture

Notes:
$p(t)_1$ – compared with cells grown in the standard medium without addition of differentiation factors;
$p(t)_2$ – compared with cells grown in differentiation medium;
$p(t)_3$ – compared with cells grown in differentiation medium after prior co-cultivation with thymocytes.

RESULTS AND DISCUSSION

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

As can be seen, the differentiation into osteogenic lineage after prior short-term co-cultivation with thymocytes increases (**Fig. 1-C**) that has also been confirmed by the results of the optical density measurement of the stains' extracts (**Table 1**). Thus our investigation has established the thymic MSCs differentiation in the osteogenic lineage *in vitro* and potentiation of this process by the thymocytes. Such effects have not been described so far *in vivo*.

Our finding of the effective adipogenic differentiation of the thymic MSCs was more expected (**Table 2**), as the capacity of thymic MSCs for adipocyte lineage may be viewed from the standpoint of their involvement in the age-related thymic involution. The obtained data indicate great similarity of the properties of the MSCs originating from various tissues and confirm their main property for multilineage differentiation.

Thus we have demonstrated that prior 24-hour contact of the thymic MSCs with the thymocytes but not with the BM cells potentiates their osteogenic differentiation considerably (**Table 1**). On the contrary, co-cultivation of the thymic MSCs with BM cells but not with the thymocytes was accompanied with the enhancement of the adipogenic differentiation (**Table 2**). However it should be noted that small number of the adipogenic progenitors of the various maturity in BM cells population can contaminate the thymic MSCs culture. These cells, versus to the osteoblasts, are present in the parenchyma of the medullary cavity and which may not be adhere to the surface during prior elimination of stromal cells and quickly proliferate in the adipogenic medium. Therefore one should be careful in considering the obtained data as such which characterize the activity of the adipogenic differentiation of thymic MSCs. Other methodological approaches are required for the solution of this problem. Therefore a conclusion relative the effectiveness of thymic MSCs' adipogenic differentiation can be made solely based on the thymocytes co-culture effects.

In the experimental conditions when the thymic MSCs and thymocytes are in a direct contact during 24 hours, the cell-cell interaction can play a certain role in the potentiation of induction of the osteogenic differentiation. However, it is not excluded that thymocytes produce certain cytokines which also contribute to the change of the MSCs properties to enhance their differentiation capacity.

Considering the common knowledge about a significant role of the stromal cell in the hematopoietic cells' function, as made evident by our data, there may be other condition when cell-cell interaction simultaneously induces significant changes in both types of the contacting cells. It should be noted that activation of the lineage differentiation of the MSCs by the hematopoietic cells may have great importance in the pathology progress when a disbalance and dysfunctions of the stromal cells can be mediated by the quantitative and functional disruption in the subpopulations of the immune cells. Further investigations along this line will promote progress in our knowledge about the systemic and local pathology in connective tissue.

CONCLUSIONS

1. ***The thymic MSCs have a typical capacity for the directed differentiation to the osteogenic or adipogenic lineage in the respective differentiation media.***
2. ***The prior 24-hour co-cultivation of the thymic MSCs with the thymocytes essentially potentiates their following osteogenic differentiation indicating the possibilities for thymocytes to influence on the thymic MSCs function.***

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The authors indicate no potential conflicts of interest.

Received: April 27, 2016
Accepted: October 26, 2016