••• КОРОТКІ ПОВІДОМЛЕННЯ ••• BRIEF COMMUNICATIONS •••

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A comparative study of the growth of primary cultured skin and lung fibroblasts from rats of different ages

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Comparative study of viability and features of growth under the same conditions of cultivation of fibroblasts of derma and lungs from rats at the age of 0,5; 1; 3; 12; 24 and 30 months has been conducted. It has been shown that curves of growth of cells quantity for both types of fibroblasts are qualitatively similar. Dependence of quantity of proliferating cells on age of animals has a bell shape with a maximum in 3 months. In both cases this maximum is observed at the end of an exponential growth phase. With age of animals proliferative activity of both types of fibroblasts decreases, that is observed in decreased level of cell adhering, of quantity increase and density of culture and increase of quantity of apoptotic and "aged" cells in cultures at the end of an exponential growth phase. In the cell culture of lung fibroblasts this decline in the ability to proliferation is expressed statistically more sharply, than in the cell culture of dermal fibroblasts.

Key words: *ontogenesis, skin, lungs, cell culture, fibroblasts.*

Сравнительное исследование роста первичных культур фибробластов кожи и лёгких крыс разного возраста

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Проведено сравнительное исследование жизнеспособности и особенностей роста в одних и тех же условиях культивирования фибробластов дермы кожи и лёгких крыс в возрасте 0,5; 1; 3; 12; 24 и 30 месяцев. Показано, что кривые роста количества клеток для обоих типов фибробластов качественно подобны. Зависимость количества размножающихся клеток от возраста животных имеет колоколообразную форму с максимумом в 3 месяца. В обоих случаях этот максимум наблюдается в конце экспоненциальной фазы роста. С возрастом животных пролиферативная активность обоих типов фибробластов снижается, что проявляется в уменьшении степени адгезивности клеток, прироста их количества и плотности культуры и повышении количества апоптических и «стареющих» клеток в культурах в конце экспоненциальной фазы роста. В культуре фибробластов лёгких это снижение способности к пролиферации выражено статистически более резко, чем в культуре фибробластов дермы кожи.

Ключевые слова: *онтогенез, кожа, лёгкие, культура клеток, фибробласты.*

Порівняльне дослідження росту первинних культур фібробластів шкіри і легень щурів різного віку

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Проведено порівняльне дослідження життєздатності та особливостей росту в тих самих умовах культивування фібробластів дерми шкіри та легенів щурів у віці 0,5; 1; 3; 12; 24 та 30 місяців. Показано, що криві росту кількості клітин для обох типів фібробластів якісно подібні. Залежність кількості клітин, що розмножуються, від віку тварин має дзвоноподібну форму з максимумом у 3 місяці. В обох випадках цей максимум спостерігається наприкінці експоненціальної фази росту. З віком тварин проліферацiйна активність обох типів фібробластів знижується, що проявляється у зменшенні ступеня адгезивності клітин, приросту їх кількості й щільності культури та збільшення кількості апоптичних і «старіючих» клітин у культурах наприкінці експоненціальної фази росту. У культурі фібробластів легенів це зниження здатності до проліферації відображене статистично більш різко, ніж у культурі фібробластів дерми шкіри.

Ключові слова: *онтогенез, шкіра, легені, культура клітин, фібробласти.*

Introduction

The differentiated fibroblasts of completely formed organism belong to three different groups which topographically are located in the derma and the non-dermal tissues of a trunk, extremities and in their distal sites (feet, palms, fingers). These groups of fibroblasts express different variants of *HOX* transcription factors genes, which determine specificity of cellular differentiation, tissue and organ morphogenesis in three different anatomic departments of a body – dermal-non-dermal, front-back and proximal-distal. Distinctions in expression of the transcription factors remain in groups of fibroblasts cultivated in vitro. It is known that these distinctions are shown, in particular, in the proliferation pattern of fibroblasts from different transcriptional groups (Chang, Chi, 2002; Rinn et al., 2006).

At the same time, possible distinctions in the proliferation pattern of fibroblasts from one transcriptional group, which participate in morphogenesis of tissues with different anatomic structure, until now weren't investigated. In principle, these distinctions can be defined as influence of the environment surrounding fibroblasts in the process of morphogenesis, and as features of fibroblasts.

Importance of the second opportunity is defined, besides pure scientific interest, first of all, that during cellular therapy it is necessary to use fibroblasts with the same properties, as in cells in the wound area (Zorin et al., 2009).

In connection with mentioned above, in this work under the same conditions of cultivation, the features of proliferation and growth of two types fibroblasts cell cultures: dermal – from skin and the non-dermal - from lungs have been investigated. Both of these cell types belong to the same transcriptional group, but form anatomical structures with different morphology.

As with an organism age proliferative activity of fibroblasts decreases (Bayreyter et al., 1995), comparative decrease of this activity in both types of fibroblasts, received from animals of different age, has been studied.

Materials and methods

Research work has been carried out on primary cultures of fibroblasts of skin from back and lung of not purebred white rats males by age of 2 weeks and 1, 3, 12, 24, 30 months living in Animal Facility of V.N.Karazin Kharkiv National University. As donors serve the animals without anatomic pathologies and deviations in behavior.

Animals were treated with sodium thiopental narcosis (by Greene, 2002), shaved a back skin site at the level of shovels and a breast, processed skin with ethyl alcohol, then in sterile conditions samples of skin (5×5 mm) and of caudal part of left lung (basis pulmonis) weighing 50 mg were cut out.

When carrying out experiments we followed recommendations of standard of biomedical ethics of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes and The Low of Ukraine "About protection of animals against ill treatment".

Tissues samples were crushed in 2 ml of the Quantum 333 "for fibroblasts" medium, containing 0,4 mg of collagenase I type, 1 mM $CaCl_2$, 0,33 mM of $MgCl_2$ and incubation was carried out within 30 minutes at 37°C (Rittié, Fisher, 2005) in the conditions of orbital mixing with a speed of 100 rev/min.

Cells which left tissues, spin down on centrifuge at 300 rpm within 5 minutes. The cell pellet was collected, sowed on the ventilated cultural mattresses (250 cm^2, Corning) in growth medium Quantum 333 and cultivation was carried out at 37°C in the conditions of 95% humidity and 5% CO_2.

The method of phase contrast on the inverted Carl Zeiss Telaval microscope with video system Photometrics Cool Snap were used for observation of cells attachment and cell culture density. Total observation time was 84 hours.

In process of cells attachment and proliferation the following parameters were determined:

Quantity of attached and apoptotic cells at the beginning of exponential growth phase (the 12th hour of cultivation).

Number and increase of cells quantity, and also cell culture density at the end of exponential growth phase (the 60th hour of cultivation).

In the middle of exponential growth phase (the 48th hour of cultivation) quantity of cells with normal morphology (active lamellar edge, 1–3 appendixes, the area of a cell 5-18 px.) and cells with morphology, characteristic for the "aged" cells (no lamellar edges, multiple appendixes, gigantism of the cell area – 20–45 px) were estimated (http://www.senescence.info/cell_aging.html).

Growth rate of cell culture was counted on tangent inclination tangent of angle to the middle of a site of exponential phase of growth curve (the 48th hour of cultivation) and expressed in terms of "cells/hour".

Table 1.

Proliferation and morphology parameters of primary cultured skin fibroblasts from rats of different ages

Age, mon.	Number of seeded cells, ×10³	Exponential phase of culture growth											
		Number of cells in the beginning of the phase (12 h.)				Number of cells in the end of the phase (60 h.)				In the middle of the phase (48 h.)			
		Adhered		Apoptotic						Morphological types of cells			Rate of culture growth, cell/hour ×10³
		×10³	%	×10³	%	×10³	Increase, %	Culture density, %		Normal		Aging	
										%	Area of the cell, px.×10³	%	Area of the cell, px,×10³	
0,5	40±1	38±2	95	1,1±0,1	2,7	351±5	823	88		100	8±1	-	-	16±2
1	40±1	36±1	90	1,8±0,1 *	4,5	318±4 *	783	80		98	11±2	2	28±1 ˣ	16±2
3	40±1	36±1	90	1,9±0,1 #	4,8	324±4 #	800	81		97	14±2	3	30±1 ˣ	16±3
12	40±1	31±1 *#@	78	3,8±0,1 *#@	12,3	244±3 *#@	687	61		74	9±1	26	26±1 ˣ	13±1*#@
24	40±1	24±1 *#@	60	5,4±1,0 *#@	22,6	180±3 *#@	650	45		42	15±3	58	42±1 ˣ	13±1 #@
30	40±1	27±1 *#@	68	7,5±1,0 *#@	28,1	146±5 *#@	440	37		38	12±2	62	27±1 ˣ	9±1 *#@

*Note. * – reliable (p<0,05) relatively to previous age; # – reliable (p<0,05) relatively to 0,5 mon.; @ – reliable (p<0,05) relatively to 3 mon.; x – reliable (p<0,05) relatively to normal morphological type of corresponding age.*

Table 2.

Proliferation and morphology parameters of primary cultured lung fibroblasts from rats of different ages

Age, mon.	Number of seeded cells, ×10³	Exponential phase of culture growth										
		Number of cells in the beginning of the phase (12 h.)				Number of cells in the end of the phase (60 h.)			In the middle of the phase (48 h.)			
		Adhered		Apoptotic					Morphological types of cells			Rate of culture growth, cell/hour 10³
									Normal		Aging	
		×10³	%	×10³	%	×10³	Increase, %	Culture density, %	%	Area of the cell, px.×10³	%	Area of the cell, px,×10³	
0,5	40±1	36±1	90	0,7±0,1	1,9	328±3	811	82	100	7±1	-	-	16±2
1	40±1	35±1	87	1,2±0,1 *	3,4	331±3	845	83	100	8±1	-	-	17±3
3	40±1	38±1	95	1,1±0,1 #	2,9	335±4	781	84	92	8±1	8	25 ±2 ˣ	15±2
12	40±1	28±1 *#	70	8,8±1,0 *#@	31,4	207±5 *#	639	52	61	13±2	39	27 ±2 ˣ	13±1
24	40±1	22±1 *#@	55	7,4±1,0 *#@	33,6	151±3 *#@	586	38	36	8±1	64	36 ±3 ˣ	12±1 #@
30	40±1	24±1 #@	60	9,5±1,0 *#@	39,6	126±3 *#@	425	32	33	11±1	67	32 ±2 ˣ	8±1 *#@

*Note. * – reliable (p<0,05) relatively to previous age; # – reliable (p<0,05) relatively to 0,5 mon.; @ – reliable (p<0,05) relatively to 3 mon.; x – reliable (p<0,05) relatively to normal morphological type of corresponding age.*

For a quantity assessment of apoptotic cells "control seeding" was done of cells in the PAA plate, class "for fluorescent microscopy". At the 12th hour of cultivation the cultural medium was deleted and fixation of cells was carried out with freshly prepared 4% solution of paraformaldehyde (in 1×PBS Dulbecco, pH 7,0). Fixation was carried out at the room temperature within 15 minutes. Then paraformaldehyde was deleted and plates bottom were washed out (duration 5 min.) with acetate buffer (pH 4,2) twice.

During this time the concentrated solution of acridine orange dye was prepared: 1 mg of dye for 1 ml of water, class "for cell culture" (it can be stored in the dark at 4°C). The fixed cells were covered for 15 min. with freshly prepared working solution of acridine orange (1 part of the concentrated solution for 9 parts of the acetate pH 4,2 buffer). Further the dye was deleted and cells were washed out with acetate buffer pH 4,2, duration 2 min. (Acridine Orange Staining …).

Then samples were dried in a stream of air of room temperature without access of light and imaging was performed on the inverted epifluorescent microscope of Carl Zeiss Telaval with laser excitation of fluorescence at 430 nm and the cutting filter 515 nm. The living (attached) cells were defined by non-fragmentized nuclei, fluorescent in green area of spectrum. Apoptotic cells – by fragmentized nuclei, fluorescent in the yellow-red area depending on an apoptosis stage (Kasibhatla et al., 2006).

The amount of cells of both types, density of culture and the area of cells field were counted according to photos of cultures, after their digital processing with programs ImageJ v.1.45s and PhotoM v.1.21.

The quantity of apoptotic cells was expressed as percentage of total amount of observed cells.

Cells were tested for mycoplasma contamination, which presence can distort results of experiment, by application MycoAlert® MycoplasmaDetectionKit test system (LonzaInc.), measuring luminescence of ATP, formed in culture from ADP, under the influence of specific enzymes of viable mycoplasmas in the presence of ADP-containing substrate of test system.

Measurement of luminescence was carried out on computerized luminometer Titertek Berthold Sirius L. Only cells suspensions with negative result of the test were used in the study.

The received results were processed statistically by the software package "Origin 6.0". Results with $p \leq 0,05$ were considered as reliable.

Results and discussion

Results of the conducted researches are presented in Tab. 1 and 2. Apparently, the general pattern of growth for both types of fibroblasts cultures and feature of their proliferation depending on age of animals are qualitatively the same.

In both cultures regardless of age of animals the lag period lasts 12 hours, then their exponential growth begins and it finishes on the 60th hour of cultivation. In the middle of this period the growth rate of both cultures is the same and does not depend on age of animals.

All other studied growth parameters of both cultures statistically significantly depend on age of animals.

So, in the period from 0,5 to 30-month age relative number of fibroblasts attached to plastic, and their quantity upon termination of cultivation, density of final cell culture and the normal cells content in it are decreased.

The sharp increase in degree of apoptosis of cultivated cells, extracted from animal tissues throughout the period of 0,5–30 months and increasing content of “aged” cells in final culture is the important reason of it. Thus the increase in the area of these cells fields, in comparison with normal, lies in standard limits.

All changes depending on age of animals in growth parameters of cell cultures in more degree are observed in fibroblasts of lungs, than in fibroblasts from derma of skin. There are observed big values of decrease with age in degree of adhesion, reduction of cells number increase and higher content of "aged" cells in final culture in limits 5–10 %. Distinctions between two cell cultures in degree of apoptosis of the cultivated cells are most expressed. During the period from 0,5 to 30-month age of animals the increase in apoptosis of fibroblasts from derma of skin makes 25,4%, and for fibroblast of lung – 37,7%.

Thus, the results can indicate lower viability of fibroblasts of lungs in comparison with dermal, especially that derived from old animals organisms (at least, in the conditions of in vitro cultivation).

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