BIOLOGICAL AND MORPHOLOGICAL CHARACTERIZATION OF *IN VITRO* EXPANDED HUMAN MUSCLE-DERIVED STEM CELLS

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Stem cells are generally characterised as clonogenic and undifferentiated cells with the capacity of self-renewal and plasticity. Over the past few years, the adult stem cells have been derived from various types of tissues including the skeletal muscle. The main goal of the present study was the isolation, *in vitro* expansion and characterisation of muscle-derived stem cells (MDSCs). Thereby obtained results showed that MDSCs have a fibroblast-like shape with a large nucleus having one to four nucleoli. The cytoplasm was transparent without any signs of vacuolisation. TEM analysis showed an ultrastructure of cells with high proteosynthetic activity. MDSCs had a large and irregular nucleus with variable number of nucleoli. The cytoplasm contained a richly developed and rough endoplasmic reticulum, prominent Golgi apparatus cisterns as well as transport vesicles containing glycogen granules and variable microvilli and filopodia. They expressed alpha-actin and desmin. Results of the phenotypic characterization showed that the analyzed cells were positive for CD29, CD34, CD44, CD90, CD105 and HLA Class I. They did not express CD14, CD45, CD235a, HLA Class II and human fibroblast surface protein. According to these results it should be emphasised that MDSCs after performing the detailed studies focused on their immunological properties and differentiation potential may be used in the cell therapy of many degenerative diseases.

Key words: muscle-derived stem cells (MDSCs), isolation, cell culture, morphological analysis, immunophenotyping.

Stem cells are generally characterised as clonogenic and undifferentiated cells derived from embryonic, foetal and adult organisms (Thomson et al., 1998; Baksh et al., 2004; Kues et al., 2005). These cells are capable of self-renewal and are unique in their potential to generate various types of tissues under proper *in vitro* and *in vivo* conditions (Barry, Murphy, 2004). Embryonic stem cells are considered to be pluripotent meaning that they are able to differentiate to cell types of all three germ layers. Moreover, they possess an unlimited capacity of symmetric divisions that provide for their long term self-renewal (Hoffman, Merrill, 2007; Nikolskii et al., 2007). For these reason, the adult stem cells represent a promising tool for tissue engineering and regenerative medicine. They are characterized by their multi-potency, asymmetric division and self-renewal ability (Shanti et al., 2007). Unfortunately, their use is restricted in many countries due to ethical considerations (Henon, 2003).

Over the past few years, the adult stem cells have been derived from various types of tissues including bone marrow, umbilical cord blood, adipose tissue, skin, periosteum, dental pulp, etc. The adult stem cells are adherent and have a fibroblast-like morphology. They are also able to produce colony forming units-fibroblast (CFU-F) when cultured *in vitro* (Caplan, 1991). These cells are heterogeneous and express a variety of surface markers including CD29, CD44, CD56, CD73, CD90, CD105, CD166, CD271, STRO-1 and Sca-1. Moreover, they are negative for haematopoietic markers CD34, CD45 and for HLA Class II (Barry, Murphy, 2004; Anokhina, Buravkova, 2007; Battula et al., 2009).

Recently, it is known that the skeletal muscle contains several stem cells populations — satellite cells, muscle-derived stem cells (MDSCs) as well as side-population cells (Usas, Huard, 2007). Satellite cells are located beneath the basal lamina *in vivo*. They represent a population of undifferentiated mononuclear cells involved in the process of myofibers enlargement. In mature organisms, satellite cells are quiescent. In the event of muscle damage, they should be activated to take on a pivotal role in myofiber reparation (Ciciliot, Schiaffino, 2010). MDSCs represent a predecessor of these satellite cells. They are characterized by a high proliferative and regenerative potential. Furthermore, MDSCs are not only able to differentiate into mesodermal cell types (e. g. myogenic, adipogenic, osteogenic, chondrogenic, endothelial, and hematopoietic lineages), but also possess the potential to break the germ layer commitment and differentiate into ectodermal lineages including neuron-like cells under proper conditions (Wu et al., 2010). Some authors even demonstrated the existence of side-population cells. Their origin and relationship to other

stem cells in skeletal muscle has been extensively studied, but remains unclear (Pavlath, Gussoni, 2005; Peault et al., 2007).

The main goal of the present study was the isolation, *in vitro* expansion and characterization of the muscle-derived stem cells as a preliminary step for their utilization in the cell therapy of degenerative diseases.

Materials and methods

MDSCs were obtained from the excisions of the femoral muscle. Sampling procedures were performed during planned surgeries, while always adhering to a patient's informed consent. All of the sampling procedures were performed in compliance with the Helsinki Declaration and were approved by the ethical committee of the Faculty of Medicine, Comenius University in Bratislava. The donors were adults without any muscular disease. The tissue samples were carefully rinsed with phosphate buffered saline (PBS, Oxoid, USA), supplemented by gentamicine at a final concentration of 200 μg/ml (Lék, Slovenia) and mechanically disaggregated with a scalpel. Subsequently, specimens were digested by pre-warmed 0.2 % collagenase type I (Pan Biotech, Germany) and 0.25 % trypsine (PAA, Austria) in a 5 % CO_2 incubator for 60 min at 37 °C. The cells and residual tissue released this way were filtered through a 70 ?m cell strainer (BD Falcon, USA) and centrifuged at 1200 rpm for 5 min. The pellet was resuspended in a culture medium containing: DMEM (PAA, Austria), 10 % foetal bovine serum (PAA, Austria), 80 μg/ml gentamicine. Finally, the cells were seeded into uncoated 60 mm Petri dishes (TPP, Switzerland) by a previously published preplate technique (Gharaibeh et al., 2008) and cultured in a 5 % CO_2 incubator at 37 °C. The culture medium was refreshed every 48 h. When the cells reached confluence they were trypsinized (0.25 % trypsine, PAA, Austria) and expanded at a seeding density of 5000 cells/mm^2. MDSCs from the third passage were appointed for growth kinetics, morphological and immunohistochemical analysis as well as for phenotypic characterization.

For the assessment of the MDSCs' growth characteristics, 200 000 cells/ml from third passage were seeded in 24-well cell culture plate (TPP, Switzerland) with a complete culture medium. The number of cells was counted in triplicate cultures every day over the period of 10 days. The morphology of MDSCs was continually analysed during cultivation using an inverted microscope Zeiss Axiovert 100 (Carl Zeiss, Germany). After the termination of cell cultivation, MDSCs were fixed by a pre-cooled methanol (Centralchem, Slovakia) for 5 min. As the next step, portions of cells were stained by Giemsa solution (Centralchem, Slovakia). After another 10 min, these were rinsed with deionized water and dried at room temperature. The second portion of MDSCs was prepared for immunohistochemical analysis of α-actin and desmin production according to the manufacturer's instructions. Antibodies were purchased from Dako Glostrup (Denmark). Observations were performed on a Nikon Eclipse 80i microscope (Nikon, Japan).

MDSCs selected for TEM analysis were fixed in 2.5 % glutaraldehyde (Sigma Aldrich, Germany). After 4 h, cells were rinsed by PBS and post-fixed 2 % osmium tetraoxide (Serva, Germany) for 2 h, then rinsed in distilled water and dehydrated in a graduated series of ethanol. Subsequently, the samples were embedded in Durcupan (Fluka, USA) and cut into semi-thin sections. The obtained sections were stained by toluidine blue (Sigma Aldrich, Germany) for 10 min and cut into ultra-thin sections. Then they were mounted on 200 mesh copper grids, double stained using uranyl acetate and lead citrate (Serva, Germany) and examined using a Philips CM 100 transmission electron microscope (Philips, Holland).

Table 1

Overview of used antibodies

Antibody	Source
Anti-CD14-PE	BD Pharmingen, USA
Anti-CD29-PE	The same
Anti-CD34-FITC	Exbio, Czech Republic
Anti-CD44-FITC	BD Pharmingen, USA
Anti-CD45-PE-Cy5	DakoCytomation, Denmark
Anti-CD90-FITC	BD Pharmingen, USA
Anti-CD105-FITC	The same
Anti-CD235a-PE	DakoCytomation, Dánsko
Anti-HLA class I-FITC	Exbio, Czech Republic
Anti-HLA DR+DP	The same
Anti-human fibroblast surface protein	Sigma Aldrich, Germany

MDSCs from the third passage were analysed by a direct and indirect immunofluorescence, according to protocols specific for each antibody. In each case, 10 000 events were acquired and analysed by a BD FACSAria flow cytometer using BD FACSDiva software (Becton Dickinson, USA). The used antibodies are shown in Table 1. FITC-conjugated donkey anti-mouse IgG antibody (Chemicon, USA) was used as a secondary antibody. Non-immune mouse isotypes served as respective controls (BD Pharmigen, USA).

Results and discussion

In the present study, MDSCs obtained from the skeletal muscle of adult donors were isolated and cultured *in vitro*. The cell culture was initiated by a preplate seeding technique due to differences in the ability of various types of cells to adhere to substrate (Gharaibeh et al., 2008; Lu et al., 2009). The choice of this method was also contingent on the fact that cells isolated directly from dissociated suspension after fluorescence-activated cell sorting or magnetic activated cell sorting seem to display a decreased regenerative ability (Cao, Huard, 2004).

During the first 48 h of cultivation, MDSCs adhered and stayed in lag phase. On the subsequent days, they started to

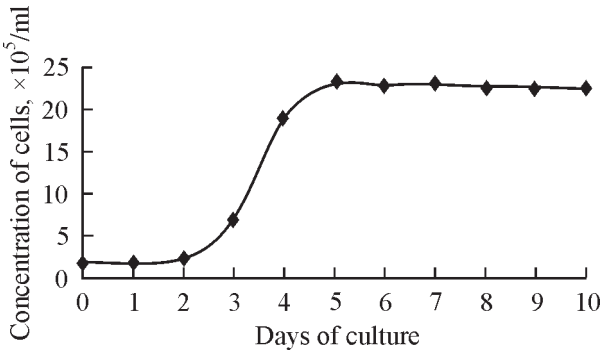

Fig. 1. Growth curves of MDSCs. Results are expressed as mean ± standard error of the mean.

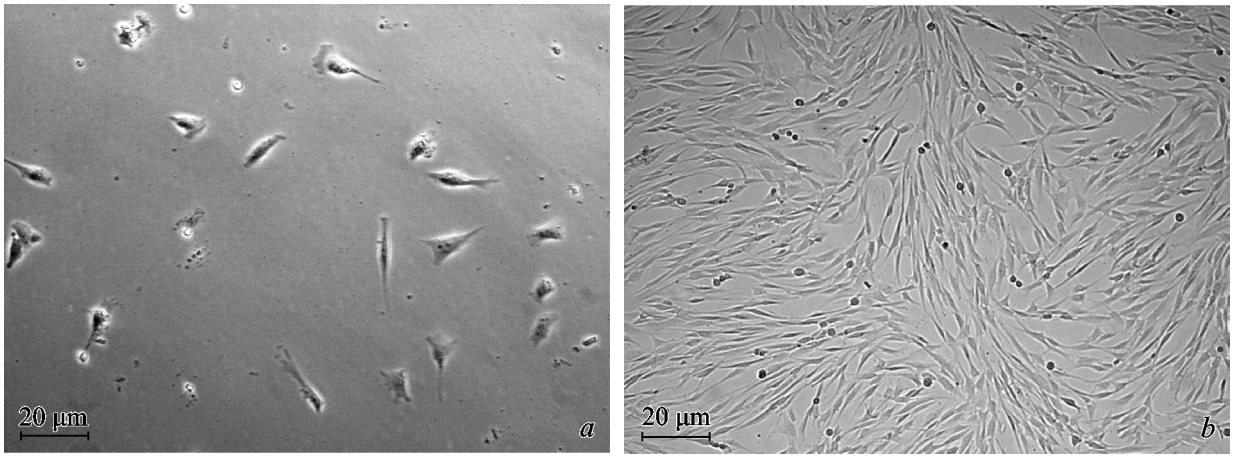

Fig. 2. Morphology of MDSCs under inverted microscope.
a — heterogenous population of cells at the initial stages of cell culture; *b* — fibroblast-like morphology in the third passage. Orig. Magn. 100×.

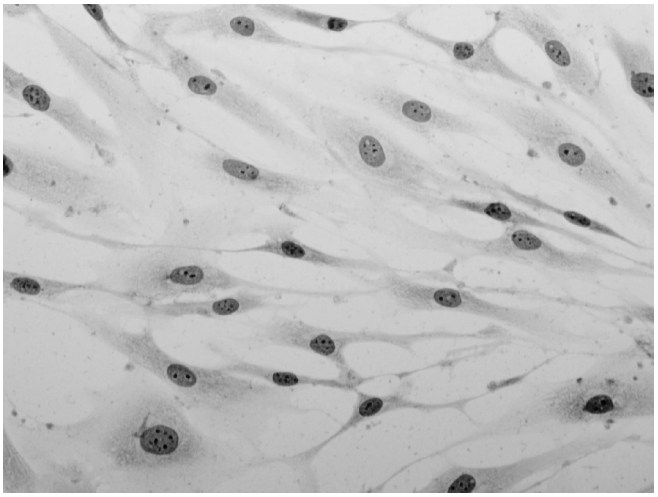

Fig. 3. Morphology of Giemsa stained MDSCs.
Orig. Magn. 400×.

proliferate as single cells or small colonies. After 8—10 days of cultivation, the cells reached a confluent layer and were sub-cultured. Contact inhibition was observed if they were not sub-cultured promptly. Similar observations were published also by other authors (Alessandri et al., 2004; Cao, Huard, 2004; Lu et al., 2009). The kinetics of proliferation of MDSCs from the third passage is presented on Fig. 1. The lag phase was almost undetectable; MDSCs adhered immediately after cell seeding. Then they started to proliferate and their number increased logarithmically. They reached the stationary phase after 5 days. Likewise, similar results were published in other papers focused on the behavior of stem cells of different origin cultured under *in vitro* conditions (Miao et al., 2006; You et al., 2009).

Primary isolated MDSCs had a bipolar to polygonal fibroblast-like morphology; they were elongated with some processes (Fig. 2). During further sub-cultivation in monolayer (up to the third passage), they maintained this morphology; no changes in their morphology or their proliferative activity were observed. Histological analysis of Giemsa stained cells showed that MDSCs had a large nucleus which was predominantly centrally situated. Nucleus had one to four nucleoli and the cytoplasm was transparent without signs of vacuolization (Fig. 3). These results are consistent with the morphological findings of several authors (Cao et al., 2003; Lu et al., 2009). Moreover, our findings correlate well with the results obtained from morphological studies of stem cells from other sources (Zuk et al., 2001; Miao et al., 2006; Moon et al., 2009).

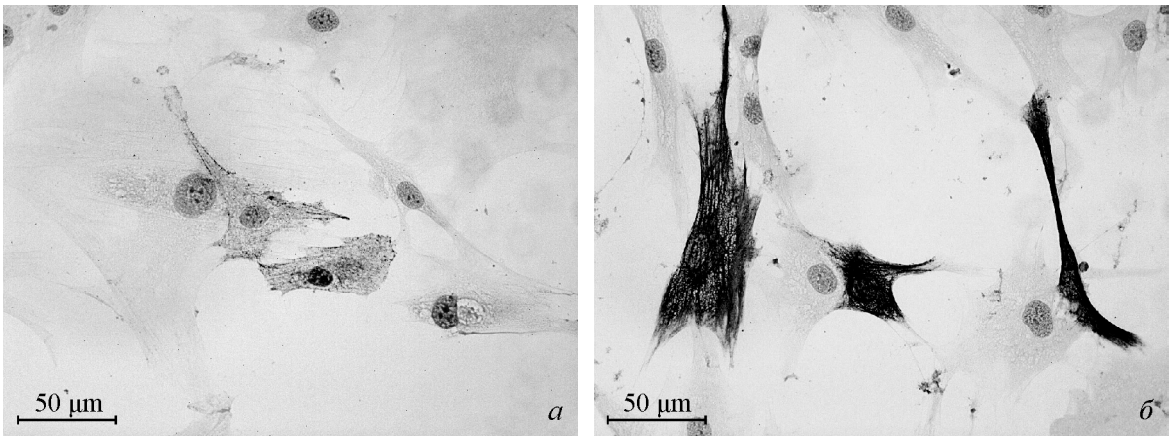

Fig. 4. Demonstration of the production: *a* — alpha-actin and *b* — desmin.
Orig. Magn. 400×.

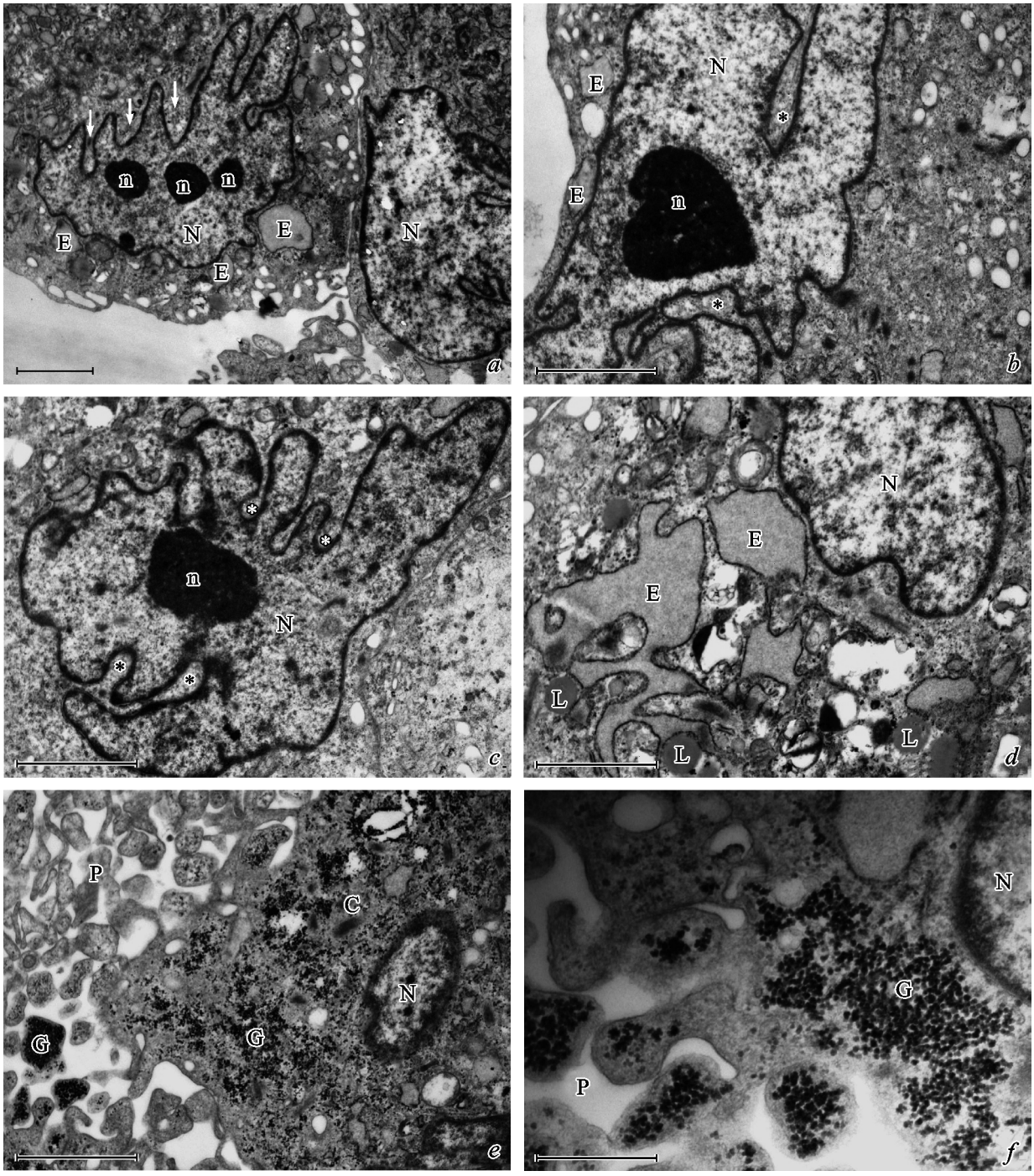

Fig. 5. Human muscle-derived stem cells in transmission electron microscope.

a — the cells had large, pale nucleus (N) with abundant euchromatine, each nucleus contains from one to three nucleoli (n). The cell nucleus ahs deep invaginations (*arrows*), a typical morphological characteristics form contractile cells. The cytoplasm is rich in well-developed rough endoplasmatic reticulum (E). Orig. Magn. 7100×. *b* — detail view on the nucleus (N) with dense, compact type nucleolus (n); nuclear envelope creates deep invaginations (*); cytoplasm contains cisterns of rough endoplasmic reticulum (E). Orig. Magn. 11 000×. *c* — large, pale nucleus (N) of human muscle-derived stem cells with large, dense nucleolus (n) and deep invaginations of nuclear envelope (*). Orig. Magn. 11 000×. *d* — detail view on the cytoplasm with extremely dilated cisterns of rough endoplasmic reticulum (E) and lipid droplets (L). A part of nucleus (N) is visible, too. Orig. Magn. 11 000×. *e* — peripheral part of muscle-derived stem cell contains mostly glycogen granules. Nearby nucleus (N) is centriolus (C). The cell create numerous processes (P), mostly microvillis and phylopodias; G — glycogen granules in cytoplasm. Orig. Magn. 11 000×. *f* — detail view on the peripheral part of muscle-derived stem cell. Part of nucleus (N), glycogen granules in cytoplasm (G) and numerous processes (P) are visible. Orig. Magn. 36 000×.

Results obtained from immunohistochemical analysis proved production of α-actin and desmin (Fig. 4). Almost 80 % were positive for desmin, what is in good correlation with results obtained by Jankowski et al. (2001). These results also indicate the capacity of MDSCs to give rise to cells of myogenic lineage (Alessandri et al., 2004).

Transmission electron microscopy showed a normal ultrastructure of cells with high proteosynthetic activity (Fig. 5, *a*—*f*). MDSCs had a large and irregular nucleus with the pre-

Table 2

Immunophenotypes of MDSCs

Analyzed marker	Test	Analyzed marker	Test
CD14	–	CD105	+
CD29	+	CD235a	–
CD34	+	Anti-HLA class I-FITC	++
CD44	+	Anti-HLA DR+DP	–
CD45	–	Anti-human fibroblast surface protein	–
CD90	++		

– — negative, + — positive ≤50 %, ++ — positive ≥85 %.

valence of euchromatine. A variable number of nucleoli were present in each nucleus. The cytoplasm contained a richly developed rough endoplasmic reticulum and prominent Golgi apparatus cisterns. Moreover, it also consisted of transport vesicles containing glycogen granules and lipid droplets. MDSCs had a variable number of microvilli and filopodia responsible for their attachment to the substrate and thus, creating contacts with neighbour cells and extracellular matrix as well as for their migration both *in vitro* and *in vivo*. The presence of processes of cytoplasm in the context of active transmigration of stem cells was described by Steingen et al. (2008).

Results of the flow cytometry analysis are shown in Table 2. The analysed stem cells were positive for CD29, CD34, CD44, CD90, CD105 and HLA Class I. They did not express CD14, CD45, CD235a, HLA Class II and human fibroblast surface protein. These results, and mainly the production of CD29, CD90 and CD105, proved their nature as mesenchymal stem cells (Barry, Murphy, 2004). Some phenotype studies have shown that MDSCs are also positive for CD34 and CD56, while being negative for CD14 and CD45 (Lee et al., 2000; Qu-Petersen et al., 2002; Alessandri et al., 2004; Jackson et al., 2007). It follows that MDSCs are considerably heterogenic and therefore, it is necessary to combine more markers for a more precise characterization. One of these markers should be the production of desmin (also analysed in our study). Jankowski et al. (2001) proved a significant expression of desmin in MDSCs. Other studies demonstrated that MDSCs are positive for MyoD and Sca-1 (Deasy et al., 2005; Arsic et al., 2008). Mentioned above open area for scientific discussion and further studies.

To sum up, *in vitro* expanded MDSCs showed the typical characteristics of somatic stem cells. After performing detailed studies focused on their immunological properties and differentiation potential, MDSCs should be used in cell therapy of a number of degenerative diseases, such as muscular dystrophy and stress urinary incontinence.

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БИОЛОГИЧЕСКАЯ И МОРФОЛОГИЧЕСКАЯ ХАРАКТЕРИСТИКА РАЗМНОЖАЕМЫХ IN VITRO СТВОЛОВЫХ КЛЕТОК, ВЫДЕЛЕННЫХ ИЗ МЫШЦЫ ЧЕЛОВЕКА

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Стволовые клетки, как правило, характеризуются как клоногенные недифференцированные клетки, обладающие способностью к самообновлению и пластичностью. За последние несколько лет стволовые клетки получены из различных типов тканей взрослых людей, включая скелетные мышцы. Основными задачами данного исследования были изоляция, наращивание in vitro и характеристика стволовых клеток, выделенных из мышцы человека. Полученные результаты показали, что мышечные стволовые клетки (МСК) имеют фибробластоподобную форму и крупное ядро, содержащее от 1 до 4 ядрышек. Цитоплазма прозрачна и без каких-либо признаков вакуолизации. Трансмиссионный электронно-микроскопический анализ показал высокую протеосинтетическую активность ультраструктуры клеток. МСК имеют большие неоднородные ядра с варьирующим числом ядрышек. Цитоплазма содержит хорошо развитый шероховатый эндоплазматический ретикулум, выступающие цистерны аппарата Гольджи, а также транспортные пузырьки, содержащие гранулы гликогена. На поверхности клеток — изменчивое число микроворсинок и филоподий. Клетки экспрессируют альфа-актин и десмин. Результаты фенотипической характеристики показали, что изученные клетки положительны по экспрессии CD29, CD34, CD44, CD90, CD105 и HLA Class I, но не экспрессируют CD14, CD45, CD235a, HLA Class II и поверхностный белок фибробластов человека. Исходя из полученных результатов следует подчеркнуть, что после проведения детального исследования их иммунологических свойств и потенциала дифференциации МСК могут быть использованы в клеточной терапии многих дегенеративных заболеваний.

Ключевые слова: мышечные стволовые клетки, изоляция, клеточная культура, морфологический анализ, иммунофенотипирование.