The use of bovine multipotent mesenchymal stem cells isolated from bone marrow and adipose tissue as sources to obtain muscle cells in vitro

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Abstract. The creation of muscle tissue with use of cell engineering technologies is currently being evaluated as one of the most promising areas in various fields of science. The aim of the work was to assess the in vitro potential of bovine multipotent mesenchymal stem cells (MMSCs) derived from bone marrow (BM) and adipose tissue (AT) to directed differentiation into muscle cells under the influence of various inducers. It was found that AT-MMSCs could differentiate into skeletal muscle cells (Sk-MCs) in response to inductive stimuli much more so than BM-MMSCs. When cultured for 28 days in the medium, supplemented with all-trans retinoic acid, bovine AT-MMSCs formed multinucleated myotubes and expressed markers of myogenesis MyoD1 and MyoG. The induction medium, including growth medium for myoblasts and horse serum, caused, in addition to myogenic differentiation, the appearance of adipocytes with lipid vesicles. The presence in the medium for differentiation of conditioned medium obtained from L6J1 rat myoblasts stimulated the directed differentiation of bovine AT-MMSCs into muscle cells, and the efficiency of its inducing action was higher. Thus, a sufficiently high potential of myogenic differentiation of bovine MMSCs has been demonstrated, these cells can be a potential source for large-scale production of Sk-MCs.

1. Introduction

In recent years, there has been a surge of concernment in the concept of creating cultured muscle tissue for food applications (also called in vitro meat or clean meat), which appeared in the early 2000s as a development perspective for livestock industry. Quickly becoming the subject of commercial interest, in vitro meat production using large-scale stem cell culturing provides researchers and industry partners with significant opportunities to use research results in the field of stem cell cultures and accumulated experience in the field of regenerative medicine [1, 2].

Innovative for agriculture and agribusiness, obtaining muscle tissue in the laboratory is determined by selecting a suitable cell source and culture conditions. Compared to the satellite cells with aging problem after several cell cycles used earlier in tissue engineering and in production of cultured meat, multipotent mesenchymal stem cells (MMSCs) have several advantages [3].

Briefly, isolated primary muscle satellite cells (myoblasts) in culture quickly proliferate and differentiate to form myotubes as early muscle fibres. Most of the published experiments with such cultures are carried out on days 3-7 after differentiation into myotubes, since myotubes in culture undergo rapid death, presumably due to spontaneous contractions [4]. These features make it difficult
to obtain a sufficient number of differentiated cells when creating large skeletal muscle constructs necessary for possible clinical applications [5]. In contrast, MMSCs are capable of self-maintenance in vitro without aneuploidy and malignization, can proliferate in culture for a long time [6] and can be induced toward myogenic differentiation in vitro.

In general, due to their main properties and characteristics, MMSCs of farm animals are biological material, promising for solving actual problems of both veterinary and virology [7, 8, 9] and agricultural, including food biotechnology [10, 11]. However, the number of published data on the characteristics of farm animal MMSCs behavior in culture is noticeably inferior to numerous studies carried out with human MMSCs [12, 13].

It is known that the influence of growth factors on proliferation and differentiation of animal MMSCs varies depending on their species and source [14, 15]. Myogenic differentiation of MMSCs can be initiated by various growth factors and compounds, including basic fibroblasts growth factor (bFGF), platelet-derived growth factor (PDGF), human epidermal growth factor (hEGF), hydrocortisone (HC) and dexamethasone (DM), all-trans retinoic acid (RA), which are added to culture medium [16-20].

Thus, the goal of this study was to determine the most promising differentiation conditions to direct MMSCs into skeletal muscle cells (Sk-MCs).

2. Materials and methods

Bovine MMSCs derived from bone marrow (BM) and adipose tissue (AT) deposited in the specialized Collection of Somatic Cell Cultures of Agricultural and Industrial Animals at the FSC VIEV were used in the study. Flow cytometric analysis showed that MMSCs were positively stained with antibodies against antigens: CD44, CD90, CD29 and were not stained with antibodies against: CD34, CD45, CD31 as previously described [21, 22]. Myogenic rat myoblast line (L6J1) was purchased from the Collection of cell cultures of vertebrates of the Institute of Cytology RAS. MMSCs and L6J1 myoblasts were used in the experiments at passages 4 and 3, respectively.

2.1. Optimization of media and conditions for directed myogenic differentiation

In the present study, MMSCs were induced to differentiate into Sk-MCs using the five different protocols (1-5) described below. In all protocols, the differentiation time was 28 days, the medium was changed every 3-4 days, unless indicated otherwise.

RA differentiation medium (1). RA (Sigma, United States) was added at a concentration of 12 μmol/l in MMSCs complete culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) with low glucose (Lonza, Belgium), 10% fetal bovine serum (FBS) (GE Healthcare, United States) and 1% penicillin-streptomycin antibiotics (Gibco, United States).

The concentration of RA was chosen based on previous study [23]. MMSCs were transferred to 24-well plates at a density of 1-10⁴ cells/cm² and then cultured in complete medium when they reach 60-80% confluency at 37°C in an atmosphere of 5% CO₂. Then RA was added to the in complete medium as follows: a) at short exposure in the first 4 days with daily medium change [24]; b) at long-term exposure for all 4 weeks.

HC + DM differentiation medium (2). Myogenic differentiation was induced using MMSCs culture medium supplemented with 5% horse serum (HS) (Lonza, Belgium), 50 μM hydrocortisone and 0.1 μM dexamethasone, as described previously [25].

SK differentiation medium (3). In this protocol, commercial growth medium SKGM-2 for Sk-MCs (Lonza, Belgium) with added 10% FBS, 2% mouse embryo extract and 1% antibiotics followed by differentiation medium. The extract was obtained from mouse embryos harvested on embryonic day 17 using the standard method of obtaining chicken embryo extract. After preparation of the embryos, the extracted brain was homogenized manually with a glass pipette with an equal volume of Hanks’ solution (PanEco, Russia), the mixture was then centrifuged at 1200 g for 20 min.

The resulting supernatant was sterilized through filters with a pore size of 0.22 μm. MMSCs were grown in SK medium for 1 week with complete replacement every 2 days. Upon reaching the 80-90%
monolayer, the cells were replaced with fusion medium for myoblasts (DMEM: F12, 1:1 mixture (Lonza, Belgium), 2% HS and 1% penicillin-streptomycin).

Co-culture system (4). Mitomycin C-treated L6J1 rat myoblasts were used as feeder cells for MMSCs. The feeder cells were seeded at 3-10⁴ cells/cm² for 1-2 days and then inactivated according to the method for STO cells [26, 27]. MMSCs were added at the same concentration as described above in the first protocol. Further, the cells were co-cultured using complete medium for MMSCs without the use of additional media with inducers.

Conditioned differentiation medium (5). Culture medium for MMSCs contained 50% medium derived from L6J1 myoblasts. To prepare the necessary volumes of the conditioned medium, cells were grown in a medium consisting of DMEM with high glucose (Lonza, Belgium), 10% FBS, and 1% antibiotics. The resulting supernatant was collected and centrifuged to remove all cells.

Experiments with cells were carried out in triplicate for each of the 2 cultures. Morphological analysis of the cells was performed using an inverted phase-contrast microscope (Carl Zeiss, Germany) with the AxioVision Rel. 4.8 software.

2.2. Real time PCR

RNA extraction from samples was performed with Quick-RNA™ MiniPrep kit (Zymo Research, United States) according to the manufacturer’s instructions. Differentiation markers were 22-kDa smooth muscle cell (Sm-MC) specific-protein (SM22α), myogenic differentiation 1 (MyoD1) and myogenin (MyoG).

PCR primers were as follows: SM22α: fwd 5'-AAGCTGGTCAATAGCCTGTATC-3', rev 5'-AGTCTTTGGTGACCCATAATC-3'; MyoD1: fwd 5'-TTTGAACACCGGACGA-3', rev 5'-GATCCAGGTCCTCAGAAGAG-3'; MyoG: fwd 5'-AGGTGAATGAAGCGAG-3', rev 5'-CCTGCAGGCGCTCTATG-3'. Samples were run at 94°C for 3 min (initial denaturation), 1 cycle; 94°C for 30 s (denaturation), 56°C for 30 s (hybridization of primers), 72°C for 45 s (elongation), 40 cycles. GAPDH housekeeping gene was used as reaction control to normalize mRNA levels.

3. Results and discussion

Based on the assumption that RA plays a key role in muscle cells development, in 2010 it was demonstrated that this compound induced differentiation of rabbit bone marrow-derived MMSCs into Sm-MCs and increased expression of smooth muscle-specific proteins [24]. However, for bovine MMSCs, short-term exposure with RA promotes their differentiation into Sk-MCs [23].

In the experiment, the effect of this compound was evaluated in the short-term (4 days) and long exposure time, which is normally used in differentiation of MMSCs to confirm their functional properties and is typically 21 days or longer. Due to the absence of specific antibodies, myogenic potential of bovine MMSCs was assessed by phase-contrast microscopy and qRT-PCR with respect to the expression of myogenic markers.

In groups of cells treated with RA (protocols 1 a and 1 b), an atypical growth of MMSCs was observed, elongating to form myotube-like structures containing more than one nucleus, which can be considered a sign of the development of myogenic differentiation (figure 1 a, b, g, h). At that, long-term induction in AT-MMSCS caused the formation of the myotubes to a far greater degree than the short-term one.

Figure 2 shows that qRT-PCR revealed low levels of gene SM22α expression in BM-MMSCs after cell differentiation according to protocols 1 a, 1 b. Compared to control, in AT-MMSC groups the expression of SM22α also remained almost unchanged after processing, with cells expressing myogenesis marker MyoD1 and MyoG. These results are consistent with observed morphological changes of cells during long-term induction with RA.
Figure 2. Expression analyses (qRT-PCR) of the myogenic markers SM22α, MyoD1, and MyoG in bovine MMSCs using five differentiation protocols (1-5) for 4 weeks.

There were no signs of differentiation in the groups of cells that were differentiated according to the second protocol, and no myotubes were detected. The mRNA values of the three genes MyoD1, MyoG and SM22α were lower than the reference values. The obtained data indicates an extremely low degree of differentiation into myoblasts.

When using culture media with myogenic factors according to protocol 3 on day 14 in all cell groups under study were identified round-shaped cells with lipid vesicles in the cytoplasm (figure 1 d, j). This result can probably be a solution to the problem of obtaining tissue structures that can be compared to in vivo muscles. It is significant that using only muscle cells as a source for cultured meat requires additional production of other components such as adipose tissue [28, 29]. The greatest degree of differentiation of BM-MMSCs into smooth muscle tissue was detected in the case of culturing using protocol 3. After 4 weeks of differentiation, cells with morphology similar to Sm-MCs were present in this group, as evidenced by the high expression of the SM22α genetic marker (figure 2 a). In the case of AT-MMSCs, cell fusion into multinuclear myotubes with a sufficiently high degree of induction in myogenic direction was registered. This conclusion can be made from the qRT-PCR results, which showed that high expression of Sk-MCs markers was detected. The obtained results are consistent with the data of others that the culturing of mouse stem cells in a medium that additionally contains 2% HS is an effective method of their differentiation into Sk-MCs [30].

For producing Sk-MCs in vitro, suitable co-culturing systems are needed that provide insulin-like growth factors and stimulate proliferation and differentiation of myoblasts. According to the literature,
blocking the cell-cell contacts responsible for the processes of myoblast fusion may be one of the promising approaches to enhance the myogenic effect of the co-culturing of L6J1 myoblasts and MMSCs [5]. Myogenic cell line L6J1 is characterized by the ability to differentiate into multinucleated myotubes, as well as the synthesis of muscle contractile proteins. Therefore, it was interesting to evaluate the paracrine effects of this cell line on bovine MMSCs through cell-cell contacts without their fusion during co-culturing. Also, as an inducer for MMSCs myodifferentiation used a medium conditioned by the growth of myoblasts, which produce molecules of metabolites and growth factors. Conditioned medium contains substrate-modifying components such as collagen and fibronectin, bFGF, insulin-like growth factors (IGF-I, IGF-II), PDGF, which is a mitogen for cells of mesodermal origin. Figure 2 shows a high level of expression of the early myogenesis marker MyoD1 in AT-MMSCs, which is characteristic of the myoblast formation stage in myogenic differentiation using the feeder layer according to protocol 4. As a result of induction using paracrine effects of L6J1 rat myoblasts according to protocol 5, in groups of AT-MMSCs were identified fusions in the elongated myotube fibers containing many cells (figure 1 l).

BM-MMSCs also changed their morphology and formed cell clusters. In this case, differentiation in both cell cultures was accompanied by expression of skeletal muscle markers. The qRT-PCR analysis revealed the strongest expression of the muscle-specific gene MyoG in AT-MMSCs (figure 2 c). It should be noted that in this group the expression of SM22α was not detected and the appearance of Sm-MCs was not observed morphologically. Meanwhile, BM-MMSCs showed only a 1.3-fold increase in the expression of the myogenic marker SM22α under culturing with the addition of conditioned medium.

Thus, optimal conditions were determined for myogenic induction of MMSCs up to myotube formation, as a promising cell source for use in tissue engineering of skeletal muscle. At this time, AT-MMSCs showed the ability to differentiate into Sk-MCs in response to inductive stimuli to a much greater extent than BM-MMSCs. The obtained results demonstrate a sufficiently high potential of myogenic differentiation of bovine MMSCs for providing the possibility of in vitro obtaining the necessary volumes of cellular material with desired properties.

Acknowledgements
The work was performed in the framework of research project No. 0578-2018-0006 “Creation of new cellular systems with desired properties based on mammalian stem cells, including from farm animals for veterinary medicine, virology, and biotechnology”.

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