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Isolation and characterization of umbilical cord-derived mesenchymal stem cells

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Abstract. Mesenchymal stem cells (MSCs) isolated from human umbilical cord have been studied by scientists for more than 10 years due to their regenerative potential. Identification of the phenotype of these cells is very important because it shows the presence of properties of stem cells. This study aims to identify the phenotype of MSCs that were obtained from the human umbilical cord. MSCs were isolated from human umbilical cord and cultured in complete α-MEM medium. The colony-forming capacities of the obtained MSCs were analyzed by colony-forming assay. The phenotype of the obtained MSCs was evaluated using flow cytometry. Isolated MSCs showed the typical morphology of MSCs and demonstrated the ability to form colonies in the CFU assay. Obtained MSCs showed the expression of surface markers of normal MSCs. Thus, the cells that were isolated from the human umbilical cord confirmed the presence of the phenotype of MSCs.
Keywords: mesenchymal stem cells, CFU, flow cytometry, phenotype, surface markers

Introduction

Mesenchymal stem cells are a heterogeneous subpopulation of multipotent cells that are isolated from synovial tissue, umbilical cord blood, perivascular areas, and human organs and tissues [1-3]. To date, interest in the use of MSCs by scientists and clinical researchers has increased, as MSCs have pronounced immunoregulatory and immunomodulatory properties, namely suppress the activity of pro-inflammatory cells of both innate and acquired immunity [4-6]. It was shown that MSCs are able to suppress the activation or induce immune cells with anti-inflammatory phenotypes [4, 5]. It was found that the immunomodulation action of MSCs occurs both through cell-cell contacts and paracrine. It is reported that MSCs secrete molecules and factors capable of activating anti-inflammatory reactions [4]. It is reported that MSCs isolated from the umbilical cord have potential in the treatment of various diseases. Before considering every type of MSCs, it is important to determine their phenotype and stem cell characteristics. Chen et.al. reported that UC-MSCs demonstrate paracrine functions that promote the formation of new vessels, alleviation of apoptosis and fibrosis, reduction of TNF-α and TGF-β levels,

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rejuvenation of cardiac properties, particularly, cardiac allografts. The immunoregulatory properties of UC-MSCs due to lowered immunogenicity demonstrated moderate elimination in comparison with BALB/c in immunocompetent mice, and also inhibition of Th1 and Th2 levels. The effect of UC-MSCs in clinical studies is being investigated for the next disease models: stable heart failure, ST-elevation myocardial infarction, acute myocardial infarction, and chronic ischemic cardiomyopathy. The authors indicated the next issues as safety and control of capacity for application in a clinical study [5, 7].

Materials and research methods

Isolation of mesenchymal stem cells from human umbilical cord

Biomaterial of the umbilical cord (UC) was taken from a healthy pregnant woman during a cesarean section at the Maternity and Childhood Center (Astana) (the report of the ethical committee is attached to the project, written informed consent was prepared before the procedure). Isolation of MSCs was carried out as described [8]. The samples were collected in phosphate-salt buffer (PSB) (Gibco, USA) and sent to the laboratory, where they were washed in PBS with 1% Anti-Anti antibiotic (Gibco, USA), cut into segments of 5 cm2, and the blood vessels were removed. Segments were placed in T25 cm2 vials and cultured in a-MEM medium with the addition of antibiotics, 1% Anti-Anti (Gibco, USA), and 10% fetal serum (Gibco, USA). Flasks with cells were incubated at 37 °C, with 5% CO2, and left alone for 7 days. Changing of medium was performed every 3–4 days. After 2 weeks, the explant PCs were removed, and the attached cells were cultured for 21 days. Then cells were passaged using 0.05% Triple (Gibco, USA). Then the cells were quantified using the trypan blue method and subcultured on serum-free medium – Stem MACS MSC Expansion (Milteniy). The morphology of MSCs was evaluated using an inverted microscope Axio Observer A1 (Carl Zeicc, Germany).

Analysis of fibroblast-like colony-forming units (CFUs)

MSCs were collected using 0.05% Triple (Gibco, USA), and the total number of cells was determined using an automatic cell counter, Bio-RadTC20. Collected cells were seeded in culture vials T25 with a density of 1000 cells/vial. After 14 days, cell cultures were washed with phosphate-buffered saline (PSB) and stained with 0.5% crystal violet in methanol at room temperature. After staining, vials were washed with FSB, dried, counted, and analyzed for fibroblast-like colonies using a stereomicroscope SZ61 (Olympus, Hamburg, Germany) [9].

Multilineage differentiation assay

For adipogenic differentiation, 1×104 cells/cm2 were seeded in a 6-well culture plate and cultured in adipogenic differentiation medium consisting of high-glucose a-MEM supplemented with 15% FBS, 0.2 mM L-glutamine, 100 μM L-ascorbic acid, 200 μM indomethacin, and 100 nM dexamethasone. After 21 days, the cells were fixed with 4% paraformaldehyde (PFA) and stained with Oil red O. For osteogenic differentiation, the cells were cultured at 90% density in osteogenic differentiation medium consisting of low glucose α-MEM supplemented with 15% FBS, 200 μM L-ascorbic acid, 10 mM glycerol phosphate, and 100 nM dexamethasone. The medium was changed twice a week for 3 weeks. Osteogenic differentiation was assessed by Alizarin red staining. For chondrogenic differentiation, cells were resuspended at 1.25×106 cells/mL in chondrogenic differentiation medium consisting of high-glucose DMEM supplemented with 1% ITS+Premix, 100 μM ascorbate-2-phosphate, 0.1 μM dexamethasone, and 10 ng/mL-β-glycerol-phosphate. To generate chondrogenic pellets, 2.5×105 cell micromasses from this cell solution

were placed in a 15 mL polypropylene tube, centrifuged at 500×g, and incubated at 37°C and 5% CO2. The medium was changed twice a week. After 3 weeks, cell pellets were collected, fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with toluidine blue [9].

Flow cytometry analysis

For flow cytometry analysis, 1×106 cells/sample were stained using the appropriate antibody kit (BD Stemflow Human MSCs Analysis Kit, USA): FITC CD90.2, PerCP-Cy 5.5 CD105, APC CD 73, PE CD44. After incubation with antibodies, cells were fixed in fixing buffer (Fix/Perm buffer, BD Biosciences) [9]. All antibodies were purchased from BD Biosciences. Analysis of cells was carried out using a flow cytofluorimeter CytoFLEX (Beckman Coulter, USA). The obtained data were processed using the program FlowJo (BD Biosciences).

Statistical data processing

Statistical processing of the results was carried out using the ANOVA test. The results of statistical processing of experimental data are presented in the form of graphs with an indication of the value of the mean square deviation.

Results

Characterization of UC-MSCs

Obtained MSCs from Wharton jelly of umbilical cord were spindle-shaped, expanded rapidly in culture, and reached confluence within 10-14 days (Figure 1B). Further analysis showed that MSCs have the capacity to form fibroblastic colonies (Figure 1C).

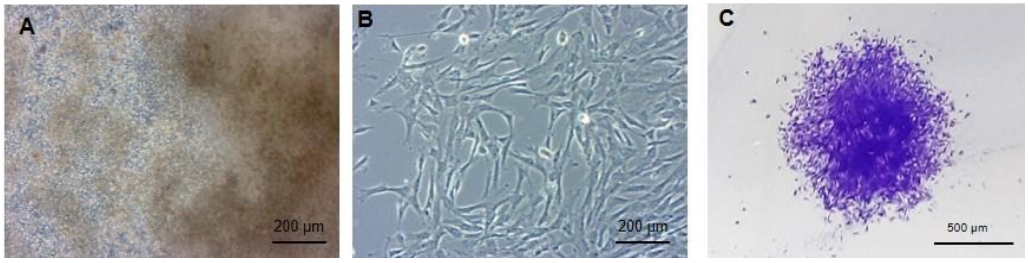

Figure 1. Isolation and characterization of the primary culture of MSCs of the human UC: **(a)** Phase-contrast image of MSCs migrating from the segments of Wharton's jelly of human umbilical cord; **(b)** Phase-contrast image of MSCs monolayer culture (passage 2); **(c)** Representative image of MSCs fibroblast colony after crystal violet staining

Obtained MSCs showed the potential to differentiate into adipocytes, osteoblasts and chondrocytes (Figure 2), indicating that they possess the self-renewal and multipotent abilities.

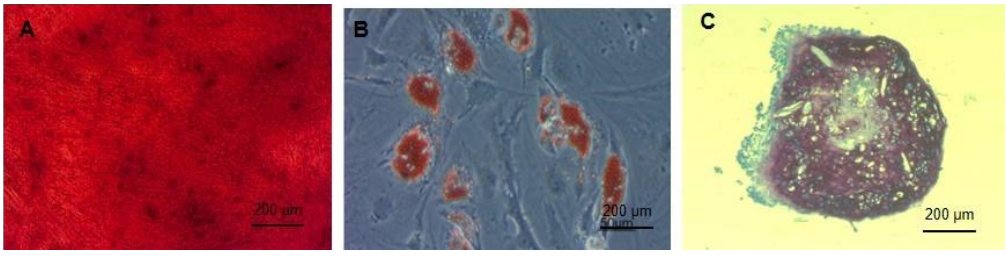

Figure 2. Multilineage differentiation of MSCs: **(a)** Phase contrast image of MSCs after osteogenic differentiation. The cells contain calcium deposits stained with Alizarin Red S. **(b)**

Phase contrast image of MSCs after adipogenic differentiation. The cells contain orange lipid vacuoles stained with Oil Red O. **(c)** Chondrogenic differentiation of MSCs in spheroid culture. Histological section of chondrogenic spherule stained with Toluidine Blue

Isolated UC-MSCs demonstrated the expression of specific markers of MSCs: CD29, CD44, CD90, and CD105 (Figure 3). Thus, these results support that our obtained cells from hUC indeed belong to MSCs.

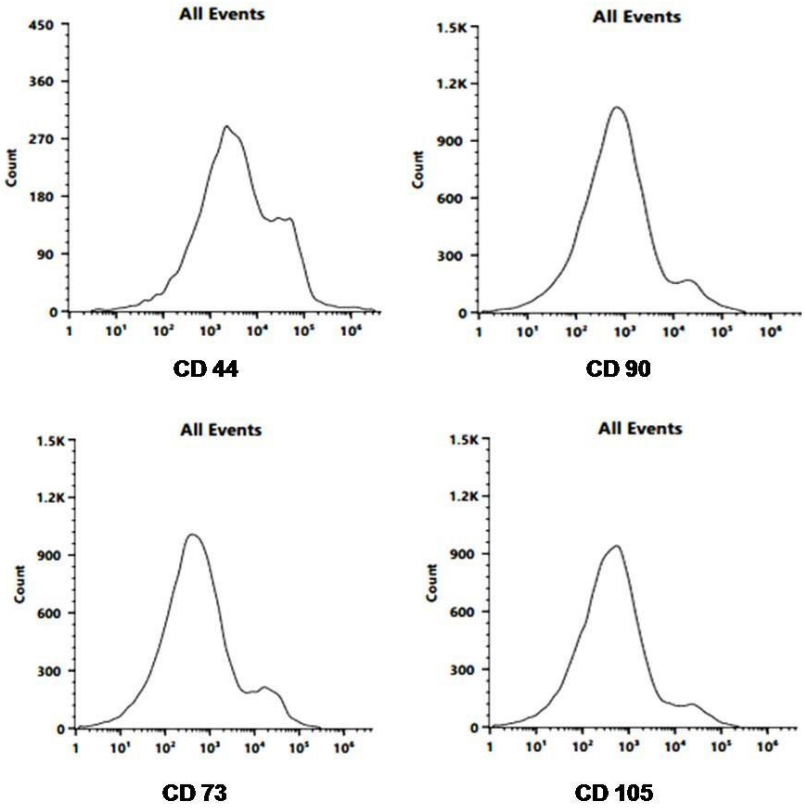

Figure 3. Flow cytometry analysis of MSCs (CD44, CD90, CD73, CD105)

Discussion

The potential of MSCs due to their immunoregulatory properties is being studied in different diseases, including autoimmune and cardiovascular diseases. Authors revealed 6 directions where MSCs' effects were found in applying for alleviation of atherosclerosis [10]. In another study, the effect of cytokine-preconditioned MSCs for decreasing the development of atherosclerosis in an animal model [11]. Before the application of MSCs for in vivo study, we examined them in the CFU assay, the multilineage differentiation assay, and in flow cytometry. The flow cytometry is the standard method for identifying if the cells meet the identity criteria according to the International Society for Cell and Gene Therapy. One of the interesting studies was focused on MSCs derived from umbilical cord (UC MSCs), adipose tissue (AD MSCs), and bone marrow (BM MSCs), and investigated their stability, expansion potential, and suitability for clinical-scale production by comparing culture in commercial media. MSCs at passages 3 and 6, and after thawing (P3), were analyzed in flow cytometry. More than 95% of cells were positive for CD73, 90, 105, and less than 2% expressed negative markers (CD34, CD45, CD11b,

CD19, HLA-DR). UC MSCs demonstrated excellent identity in both media, with positive markers expressed near 100% and negative markers well below 2%. AD MSCs showed in Stem MACS medium high positive and low negative expression, while in Power Stem medium, there was high expression of positive markers, but some samples exceed 2% for negative markers. BM MSCs had a drop in CD90 and CD105 marker expression after culturing in Power Stem medium, with very high negative results (45% approximately). This indicated very poor performance of the cells in this medium. Authors found that Stem MACS MSC Expansion is the most suitable and consistent medium for cell growth. These cells, grown on selected media, were not contaminated with other cell types and fully meet the standard criteria by the International Society for Cell and Gene Therapy (ISCT). The CD73 factor is responsible for regenerative function, conversion of adenosine-monophosphate to adenosine, and contributes to anti-inflammatory signaling in MSCs. The CD90 is responsible for the ability to adhere, migrate, and differentiate, meaning the function of cell-cell and cell-matrix interaction in MSCs. The CD105 participates in vascular regeneration and tissue healing, a co-receptor of TGF beta (transforming growth factor) [9].

Colony-forming unit assay (CFU) is the standard method to determine the cell proliferation capacity and clonogenic potential of MSCs. Authors, by using this assay, found that UC MSCs had a similar number of cells in both Stem MACS and Power Stem with high clonogenic potential. If AD MSCs performed better proliferation in Stem MACS media, with the average CFU counts, however, BM MSCs were slightly higher in Stem MACS, but not statistically significant (P=0.13). The colony-forming unit assay was performed on MSCs of passage 2. The seeding density was the next: UC-MSCs - 4 cells/cm^2, AD-MSCs - 20 cells/cm^2, and BM-MSCs - 100 cells/cm^2. Authors found that UC MSCs had a similar number of cells in both StemMACS and PowerStem with high clonogenic potential (the highest CFU count overall: average 366 ±154 colonies). However, AD MSCs performed better in StemMACS, with average CFU counts of 97 ± 18 colonies. In addition, BM MSCs were slightly higher in StemMACS, but not statistically significant (P=0.13), with the lowest colony overall 16 ±3 CFUs [12, 13]. Other researchers also focused on studying how different sources of mesenchymal stromal cells (MSCs) affect the ex vivo expansion of hematopoietic stem/progenitor cells (HSPCs) derived from umbilical cord blood by comparing the MSCs from three sources (bone marrow, adipose tissue, and umbilical cord matrix). The study also examined whether replacing fetal bovine serum (FBS) with human platelet lysate (HPL) during the expansion of MSCs. BM- and AT-derived MSCs expanded with FBS improved the UCB HSPC expansion and maintenance of primitive subsets, while UCM-derived MSCs and HPL-based cultures may compromise these outcomes [14].

In regenerative medicine, bone marrow-derived mesenchymal stromal cells are becoming increasingly important. Many scientists study the MSCs' therapeutic value, but some issues regarding the expansion were raised. For the cell therapy, fetal bovine serum usage causes challenges, including safety problems and the variety of media compositions. This is why serum-free media (SFM) usage is becoming more diverse, studying the specificity of media for cell growth. To identify the most suitable media, six commercial SFMs were analyzed at two different densities for BM-derived MSCs culturing, where differences in marker expression of MSCs were seen after altering the time, yield, colony formation, differentiation, and immunosuppression. This study showed the possible replacement of animal-derived serum for safer conditions and clearer results. Overall, Rooster Nourish, Rooster Nourish-MSC XF, StemMACS-MSC XF, and MSC NutriStem XF media showed the highest expression of positive markers (>98%) and the lowest expression of negative markers (<1%) in flow cytometry. This demonstrated that these commercial media are consistent across seeding density variations and cryopreservation influence.

BM-MSCs were also examined in CFU analysis. It was found that serum-containing medium had the highest number of colony formation, with dense, large, and numerous colonies. BM-MSCs cultured in the Rooster Nourish media showed a meaningful number of CFU as well, while other SFM/XFM, especially MSC NutriStem, showed significantly reduced colony formation [15, 16]. It was reported that mesenchymal stem cell markers like CD73 and CD90 are usually used to reflect the original state of cells. The authors analyzed cells from periosteum and cartilage, finding that >95% of cultured cells expressed CD73 and CD90, regardless of their original tissue or culture conditions. Nevertheless, it was found that freshly isolated cells do not have these markers, suggesting that the expression of CD73 and CD90 is possible during in vitro culture, but not in vivo. Therefore, the expression of MSCs' markers was not connected to reflecting their natural identity or functional state.

Authors found that after in vitro cultivation, cell populations acquired similar surface phenotypes with high expression of CD73 and CD90, with the loss of CD34 [17, 18]. It was reported that cord blood-derived mesenchymal stromal cells (CB-MSCs) are emerging as a promising option to support immune tolerance and reduce the need for traditional immunosuppressive drugs. Because of their unique anti-inflammatory and anti-apoptotic properties, they can be considered for clinical use. The GMP-compliant (Good Manufacturing Practice) process to produce and bank clinical-grade material for use in advanced therapy medicinal products (ATMPs) was considered. Between 2012 and 2023, 263 CB-MSC doses were safely administered to 40 patients with conditions like Graft-versus-Host Disease, nephrotic syndrome, and neonatal bronchopulmonary dysplasia. The authors found no serious side effects, and most patients showed positive outcomes. CB-MSCs were analyzed in flow cytometry for expression of 3 positive and 1 negative marker. From the results, CB-MSCs were characterized by strong expression of CD90 and CD105 and absence of CD45, which was used to identify any contaminating non-MSC cells. Also, the cells showed strong purity, with an average of 97.3 ± 2.7% expressing key MSC markers CD90, CD105, and lacking the blood cell marker CD45. Contaminating cells were nearly absent, and viability was high, with over 92.7 ± 4.6% of the cells alive and healthy at the time of testing. Even after thawing, the cells maintained good viability.

Additional tests confirmed that the cells consistently expressed other important markers like CD73, and all safety checks, like sterility, absence of mycoplasma or viruses, and normal karyotype, were successfully passed. These results highlight the reliability and clinical readiness of the MSC(CB) produced under strict GMP standards. Also, the colony-forming units' assay showed that CB-MSCs formed 17.9 ± 17.4 colonies [19, 20]. It was revealed that the potential of MSCs derived from the umbilical cord blood can be considered for skin diseases like psoriasis. Authors exhibited in vitro and in vivo studies on the application of MSCs and derived exosomes for psoriasis disease [21]. Another work of the same author presented the findings on the use of cytokine preconditioned MSCs that were isolated from human umbilical cord blood for the alleviation of psoriasis in a mouse model.

The flow cytometry analysis of hUCB-MSCs also showed positive expression of CD90, CD73, and CD105 markers [22]. Another study compared the osteogenic capabilities of MSCs of adipose tissue and Wharton's jelly of the umbilical cord, which were cultured under identical differentiation conditions to determine which MSC source is more effective for bone regeneration. Authors evaluated the osteogenic differentiation using established biomarkers, including calcium deposition (Alizarin Red S staining), alkaline phosphatase (ALP) activity, and the secretion of osteoprotegerin (OPG) and osteocalcin (OC). The low OPG and high OC levels in both cell types indicated the progression to an advanced osteogenic differentiation stage.

However, WJ-MSCs demonstrated greater OC secretion than AT-MSCs. These findings confirmed that both AT-MSCs and WJ-MSCs possess the osteogenic potential, though the differences in biomarker expression suggested that AT-MSCs may have reached a more mature differentiation stage by day 21 [23, 24]. Also, drawing on a wide range of previous studies, offers a comprehensive overview of how various cell types, including macrophages, endothelial cells (ECs), nerve cells, and periodontal cells, affect the osteogenic differentiation of mesenchymal stem cells (MSCs).

In addition to serving as a valuable reference for developing multicellular co-culture strategies in osteogenesis research, the article also provides a solid foundation of basic knowledge on the mechanisms and factors involved in MSCs' osteogenic differentiation. The osteogenic differentiation of MSCs progresses through distinct stages: proliferation, commitment to the osteoblast lineage, and matrix mineralization. This complex process is orchestrated by a network of transcription factors, including Runx2, Osterix (OSX), DLX5, and ATF4, and is regulated by key signaling pathways such as Wnt/β-catenin, BMP, Notch, FGF, and Hedgehog. Among these, Runx2 is particularly critical, serving as a central hub that integrates signals from multiple pathways to drive osteoblastic differentiation [25, 26]. In this review authors reported on chondrogenic differentiation in mesenchymal stem cells, in comparison with the differentiation potential of cancer stem cells (CSCs). The complex biological mechanisms are involved in this process, such as signaling pathways: TGF-β/Smad, BMPs, Hedgehog, Notch, Wnt, and FGF, all of which are converged in the regulation of the SOX9, the master transcription factor in chondrogenesis. It was indicated that a comprehensive multi-dimensional scope—addressing not only canonical molecular pathways but also non-coding RNAs (miRNAs, lncRNAs, circRNAs) and biophysical factors like oxygen tension, mechanical stress, and substrate stiffness, which can shape the fate of MSCs [27, 28]. It was reported that MSCs can be considered as a promising tool in tissue engineering, but their tendency to lose potency in the creation of cartilage that isn't fully comparable to native tissue [29, 30].

Our obtained results of all experimental analyses confirmed that the cells isolated from the Wharton's jelly of human umbilical cord supported the characteristics of mesenchymal stem cells: showed a fibroblast-like morphology, demonstrated the differentiation potential into three cell types (osteocytes, adipocytes, and chondrocytes), and reliably expressed the positive surface markers - CD44, CD90, CD73, and CD105.

Conclusion

Thus, the cells that were isolated from the human umbilical cord confirmed the presence of the phenotype of MSCs.

Author Contributions

V.O., A.S. - Conception and design; A.S., A.I. - Collection and assembly of data; A.S., A.I., V.O. - Data analysis and interpretation; A.S. -Manuscript writing; V.O. - Final approval of manuscript.

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Conflicts of Interest

All authors have completed the ICMJE uniform disclosure form. All authors have no conflict of interest to declare.

Compliance with ethical standards

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Кіндік бауынан мезенхималды бағаналы жасушаларды алу және сипаттау

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Аңдатпа. Ғалымдар адамның кіндік бауынан бөлінген мезенхималық бағаналы жасушаларының (МБЖ) регенеративті потенциалын 10 жылдан астам зерттеп келеді. Бұл жасушалардың фенотипін анықтау өте маңызды, өйткені ол бағаналы жасушаларының қасиеттерінің болуын көрсетеді. Бұл зерттеудің мақсаты адамның кіндік бауынан алынған МБЖ-ның фенотипін анықтауы болып табылады. МБЖ адамның кіндік бауынан бөлініп, толық

α-МЕМ ортада өсірілді. Алынған МБЖ-ның колония түзу қабілеті колония түзуші талдау арқылы талданды. Алынған МБЖ фенотипі ағындық цитометрия көмегімен бағаланды. Алынған МБЖ типтік морфологиясын көрсетті және CFU талдауында колониялар құру мүмкіндігін көрсетті. Алынған МБЖ қалыпты МБЖ беттік маркерлерінің экспрессиясын көрсетті. Осылайша, адамның кіндік бауынан алынған жасушалар МБЖ-ның фенотипінің болуын растады.

Түйін сөздер: мезенхималды бағаналы жасушалар, CFU, ағындық цитометрия, фенотип, беттік белгілер

Выделение и характеристика мезенхимальных стволовых клеток из пупочного канатика

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Аннотация. Регенеративный потенциал мезенхимальных стволовых клеток (МСК), выделенных из пуповинной крови человека, изучается уже более 10 лет. Определение фенотипа этих клеток имеет важное значение, поскольку указывает на наличие свойств стволовых клеток. Целью данного исследования было определение фенотипа МСК, полученных из пуповинной крови человека. МСК были выделены из пуповинной крови человека и культивированы в полной среде α-МЕМ. Способность к колониеобразованию полученных МСК была проанализирована с помощью анализа CFU. Фенотип полученных МСК был оценен с помощью проточной цитометрии. Полученные МСК имели типичную морфологию и продемонстрировали способность образовывать колонии в анализе CFU. Полученные МСК экспрессировали поверхностные маркеры МСК. Таким образом, клетки, выделенные из пуповинной крови человека, подтвердили принадлежность к МСК.

Ключевые слова: мезенхимальные стволовые клетки, CFU, проточная цитометрия, фенотип, поверхностные маркеры

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