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Isolation of Multipotent Mesenchymal Stromal Cells from Cryopreserved Human Umbilical Cord Tissue

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Umbilical cord stroma is an easily available, convenient, and promising source of multipotent mesenchymal stromal cells for regenerative medicine. Cryogenic storage of umbilical cord tissue provides more possibilities for further isolation of multipotent mesenchymal stromal cells for autologous transplantation or scientific purposes. Here we developed a protocol for preparation of the whole umbilical cord tissue for cryogenic storage that in combination with the previously described modified method of isolation of multipotent mesenchymal stromal cells allowed us to isolate cells with high proliferative potential, typical phenotype, and preserved differentiation potencies.

Key Words: *multipotent mesenchymal stromal cells; umbilical cord; cryopreservation; cell culture*

Multipotent mesenchymal stromal cells (MSC) from human umbilical cord (HUC) due to their unique biological properties are more and more widely used in various fields of regenerative medicine [6-8,15]. Numerous recent reports describe new protocols of isolation and culturing of MSC from the whole umbilical cord or some its compartments (Wharton's jelly, perivascular areas, amnion, and subamniotic structures) obtained within the first hours after delivery [1,2,5,9,11,13-16]. The possibilities of cryogenic HUC storage for deferred isolation of cells received less attention [3,4,12]. However, storage of frozen biomaterial can be useful for creation of a bank of extraembryonic tissues (*e.g.*, as the source of autologous MSC for regenerative medicine or isolation of induced pluripotent cells) and for prospective biomedical studies of rare inherited or acquired diseases.

Despite some variations in the freezing medium composition, use of fetal calf serum (FCS), umbilical cord blood plasma, or commercially available formulations, freezing protocols, and times of cryogenic storage (from 2 weeks to 5 years), most studies have generally confirmed the possibility of MSC isolation from frozen umbilical cord tissue [3,4,12]. Subsequent comparative analysis of functional parameters of cells isolated from fresh or frozen/thawed tissue revealed no significant differences in MSC yield per tissue mass unit, expression of surface markers, or multilineage differentiation potencies. At the same time, cryogenic storage of HUC tissue adversely affected viability of isolated cells (especially after enzymatic isolation) and their proliferation capacity [3].

Our aim was to develop reproducible conditions of HUC tissue preparation for cryogenic storage and subsequent MSC isolation and culturing.

MATERIALS AND METHODS

Isolation of the biological material. Samples of HUC (n=5) were collected from healthy women after vaginal delivery at the Obstetric Department of the V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology (all women signed informed consent). After delivery, the fetal end of the umbilical

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cord was clumped with two clamps and cut between the clumps. The third clump was applied near the placenta. The resultant 20-30-cm fragment of the umbilical cord was packed in a zipped plastic bag, placed in a transport container, and transferred to the laboratory within 6-12 h for further processing.

Preparation of the tissue to cryogenic storage. The tissue was processed as described previously for the isolation of MSC fresh HUC tissue [1]. After removal of the residual blood with sterile gauze, the HUC was sterilized with antiseptic Okteniderm (Shulke & Mayr) and cut into fragments (~2×2×2 mm). The tissue fragments were transferred in centrifuge tubes and intensively washed with sterile saline.

Choosing the time of exposure with cryoprotectant. The tissue fragments were divided into groups and transferred in sterile 3.6-ml Corning cryotubes (8-10 fragments per tube). Then, 1.5 ml cold MSC culture medium (see below) with elevated serum concentration (20%) and the same volume of the same medium containing 20% DMSO were added to the tube. The cryotubes were placed in an ice bath for 5, 15, 30, 45, and 60 min, respectively.

Programmable freezing. The material obtained was frozen in accordance with standard procedures of accepted in Cord Blood Bank by the program designed to freeze hematopoietic stem cells of the umbilical cord blood. After freezing to a temperature -90°C, tube was transferred to a quarantine Dewar flask and stored in liquid nitrogen vapor at temperatures below -160°C.

Thawing of the tissue and isolation of MSC. At the end of cryogenic storage time (2-3 months), the tubes with frozen tissue fragments were thawed in a water bath at 37°C. The content was transferred to sterile centrifuge tubes, washed from the cryoprotectant for 30 min in an excess of cold culture medium, and centrifuged at 100*g* in a start-stop mode.

Isolation, culturing, and subculturing of MSC. MSC were isolated by the method described previously in detail [1]. The tissue fragments washed from the cryoprotectant were incubated in collagenase IV solution (Sigma) in culture medium for 30-45 min on ice. DMEM/F-12 supplemented with 100 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Life Technologies) served as the culture medium for MSC. Then, the tissue fragments were washed from the enzyme, transferred to 35-mm Petri dishes, spread on the surface under stainless steel grids. The volume of the culture medium was brought to 2 ml and the dishes were placed in a CO_2 incubator (5% CO_2, 37°C, 100% humidity). The medium was first changed in 16-18 h after cell isolation and then twice a week. Cell migration, growth, and morphology were evaluated by phase-contrast microscopy (Axiovert 40, Nikon).

In 2 weeks, the grids and remnants of the explants were removed. One week later, the cultures were washed with sterile physiological saline and removed with trypsin-EDTA (Life Technologies). The cell suspension was resuspended in fresh culture medium, diluted 1:5 or 1:10, and seeded to Petri dishes or flasks (Corning). After attaining confluence, the procedures were repeated.

Flow cytofluorometry. For evaluation of the expression of surface differentiation markers, passage 3 cells were removed with trypsin-EDTA, washed from the enzyme by centrifugation in a great volume of culture medium. The cell suspension was divided into 50-μl aliquots (10^5 cells) and incubated at room temperature with FITC- or phycoerythrin (PE)-conjugated antibodies to the corresponding clusters of differentiation markers (CD): CD13, CD29, CD34, CD44, CD45, CD54, CD71, CD73, CD90, CD105, CD117, CD146, HLA-ABC, HLA-DR (Beckman Coulter or BD Pharmingen), and VEGF-R2/KDR (R&D Systems) in concentrations recommended by the manufacturer. Non-immune antibodies of the corresponding class were used as the negative control. The cells suspensions were analyzed on a FACSCalibur flow cytofluorometer (BD) using CellQuest Pro software; 10,000 cells were analyzed in each sample.

Targeted differentiation of MSC. Passage 3 MSC cultures were incubated with relevant inductors as described previously [1.10] or commercial kits StemPro (Life Technologies) to confirm adipogenic and osteogenic potencies of MSC. In 2-4 weeks, the cultures were fixed in 1% buffered formalin. Differentiation was assessed by the presence of lipid droplets (Red Oil O staining), alkaline phosphatase activity, or mineralized matrix (alizarin red staining).

Statistical analysis. Significance of differences between the groups was evaluated using parametric (*t* test for dependent and independent samples) and non-parametric tests (Wilcoxon's test) (Statistica 10.0, StatSoft). The differences were significant at $p<0.05$.

RESULTS

The lots of culture medium components, sera, and collagenase were the same for cell isolation from fresh umbilical cord tissue [1]. However, processing of frozen/thawed tissue fragments under conditions that were optimal for fresh samples was associated with excessive tissue disaggregation leading to accumulation of viscous collaged degradation products. In light of this, concentration of collagenase was 2-fold reduced (to 0.05%) at the same time of incubation o tissue fragments in cold enzyme solution (30-45 min). Other stages of cell isolation and culturing were not modified.

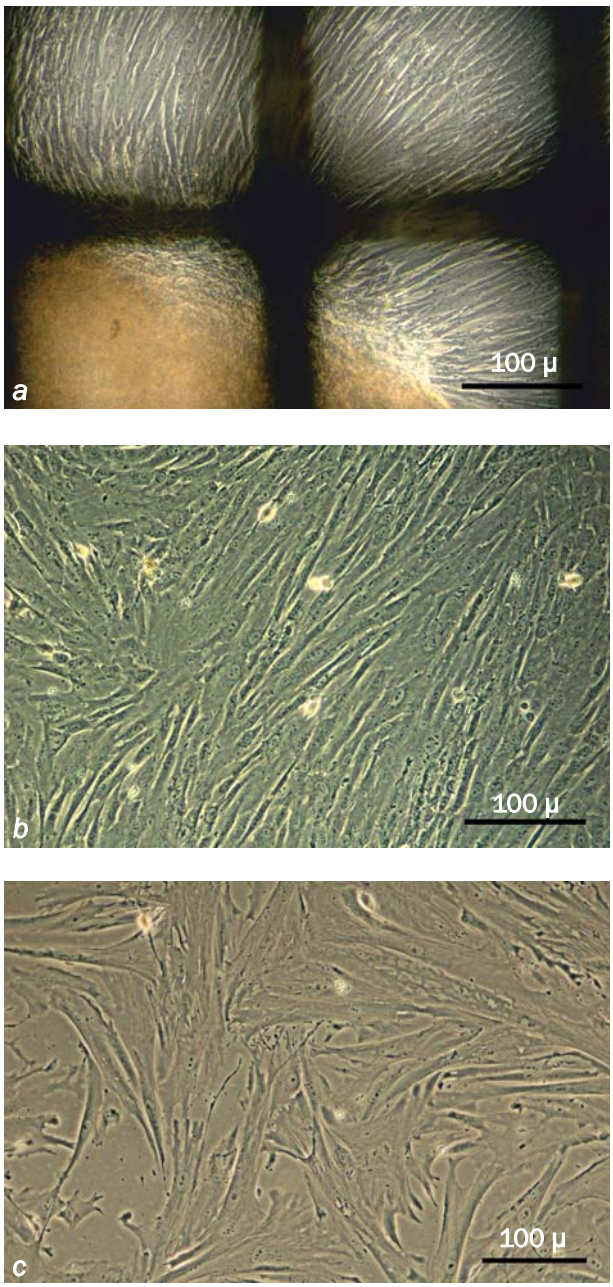

Fig. 1. Culture of MSC isolated from frozen/thawed HUC tissue. Phase contrast. *a*) Cell migration from tissue fragment on day 9; *b*) confluent culture, passage 3; *c*) aging culture, passage 11.

As in the case of MSC isolation from fresh HUC tissue, active migration of small spindle-shaped cells was observed as early as on days 7-9 of culturing (Fig. 1, *a*). MSC cultures were obtained from all examined tissue samples irrespective of the time of incubation with the cryoprotectant (5-60 min). However, the best results were obtained after incubation in DMSO solution for 20-45 min. The cells actively proliferated and migrated over the next two weeks and almost completely covered the surface of culture plastic forming a homogenous monolayer. In both cases, MSC used both types of matrix — culture plastic and stainless steel grids for their growth (data not shown).

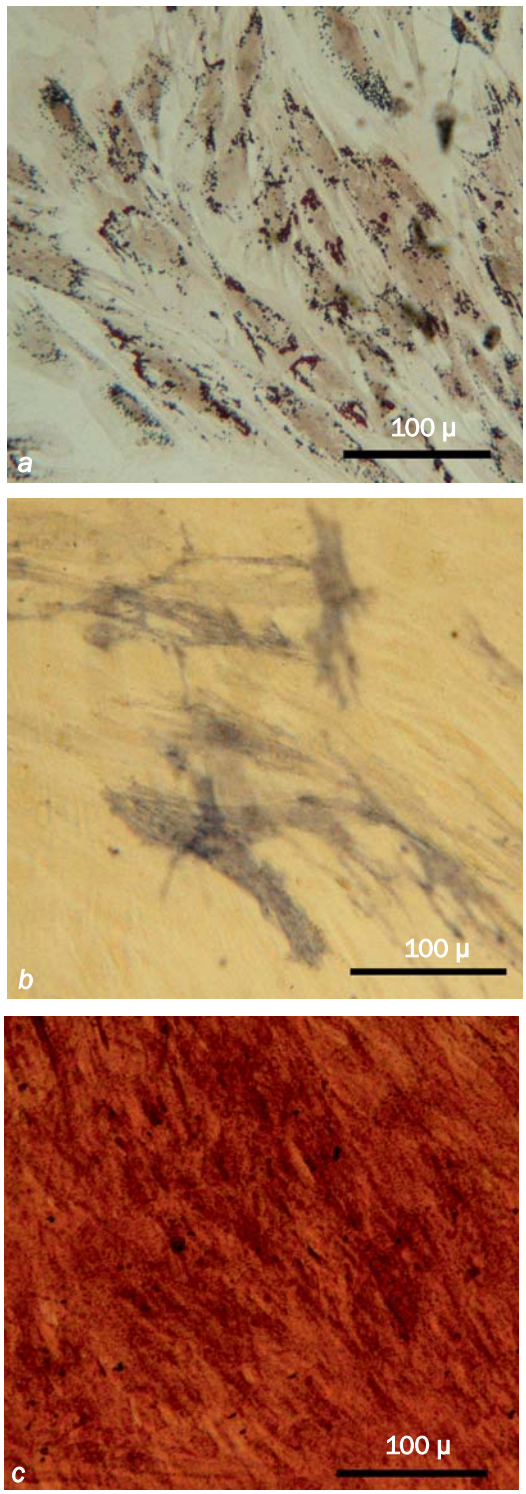

Fig. 2. Phenotyping of MSC isolated from fresh (green curves) or frozen/thawed (red curves) HUC tissue (passage 3). Flow cytofluorometry. Results of one experiment from a series of representative experiments are presented.

Proliferative activity of MSC isolated from fresh and frozen biological material did not differ (in this

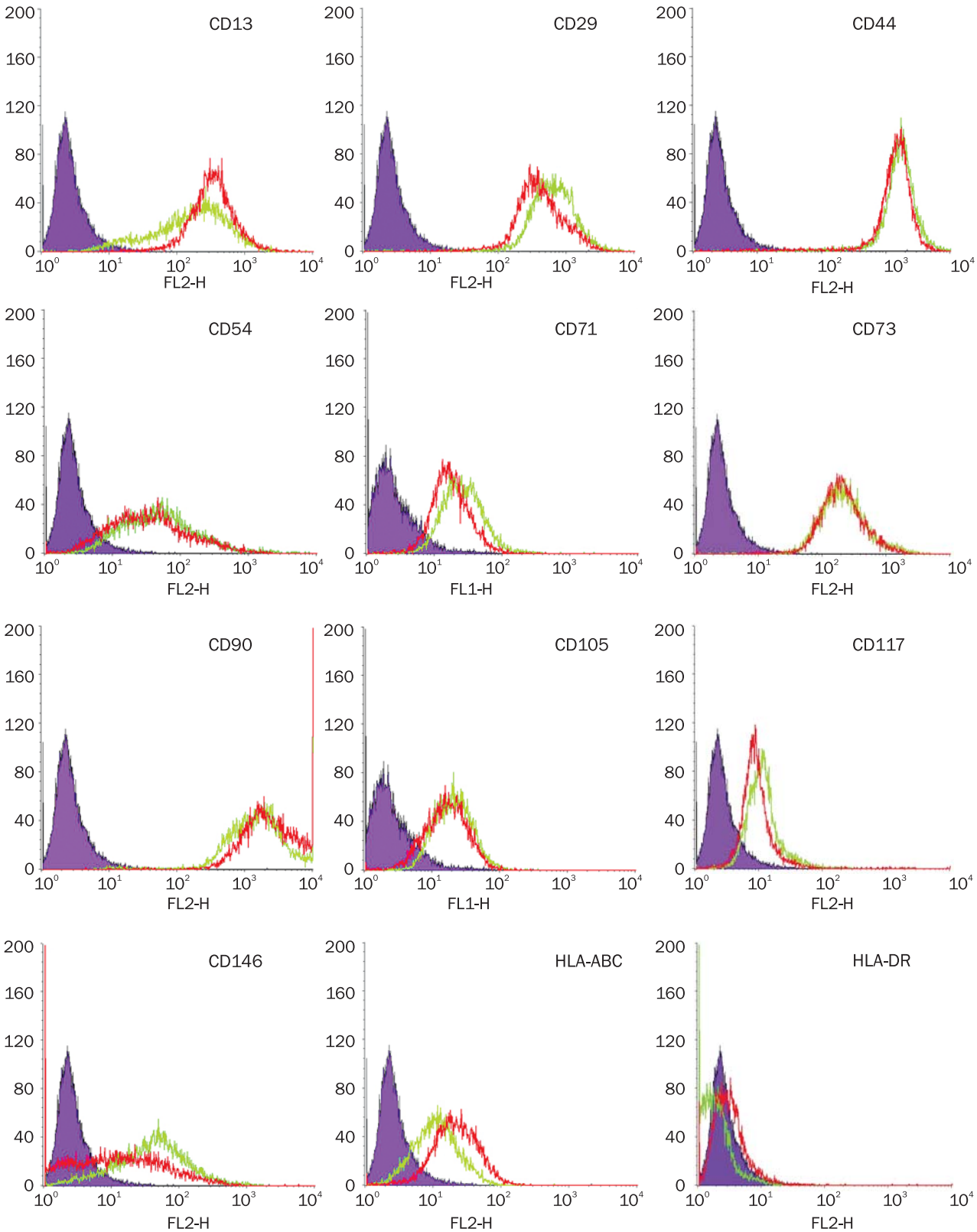

Fig. 3. Adipogenic and osteogenic differentiation of MSC isolated from frozen/thawed HUC tissue (passage 3). Light microscopy. *a*) Visualization of lipid droplets, Red Oil O staining; *b*) detection of alkaline phosphatase activity; *c*) mineralized extracellular matrix stained with alizarin red.

study, we used the same HUC samples): the cells passaged 1:5 and 1:10 almost simultaneously attained confluence by days 7-9. Until passage 9, the cell morphology (Fig. 1, *b*), density of the formed monolayer (5.2±1.6 and 3.8±0.9×10^4 cells/cm^2, p>0.05), mean population doubling time (78.8±19.5 and 51.1±4.1 h, p=0.004), mean number of doublings (2.23±0.48 and 3.29±0.26, p=0.007), and intervals between passages remained practically unchanged. During this period (passage 1 monolayer culture on a 35-mm Petri dish was taken as the initial culture), the cell passed 20 and 30 population doublings, and their total number reached 1.1×10^{12} and 8.3×10^{14}, respectively. Only on passages 10-11, signs of aging appeared in cultures: accumulation of highly flattened cells, multinuclear cells (Fig. 1, *c*), deceleration of proliferation (mean doubling time>100 h), and lengthening of the intervals between passages to 12-14 days. Over the next 2-3 passages, the MSC cultures lost their proliferative activity and almost completely degraded.

Flow cytofluorometry showed that phenotype of MSC isolated from cryogenically stored umbilical cord tissue did not differ from the phenotype of cells isolated from fresh samples of the same HUC at the early (3-4) and later (5-6) passages (staining and analysis of cells was performed under identical conditions at the same instrument adjustment parameters). Most cells expressed CD90, CD105, CD73, CD13, CD29, CD44, CD54, CD71, CD117, CD146, and HLA-ABC (Fig. 2). Neither markers of cells of hematogenous origin (CD34, CD45, and HLA-DR), nor endothelial cell markers (CD309/VEGF-R2/KDR) were detected.

Evaluation of adipogenic and osteogenic differentiation capacities of MSC showed that cryogenic storage of HUC tissue did not impair functional characteristics of these cells. Culturing for 2-3 weeks in media containing appropriate inductors led to the appearance of numerous cytoplasmic lipid inclusions in almost 100% cells (Fig. 3, a); alkaline phosphatase was expressed in ~50-60% cells (Fig. 3, *b*). Longer (4 weeks) osteogenic differentiation led to the appearance of fragments intensively stained with alizarin red in cultures indicating the formation of mineralized extracellular matrix (Fig. 3, *c*).

The study showed that optimized protocol of MSC isolation from HUC tissue developed by us could be successfully used for fresh and cryogenically preserved biological material. Comparative analysis of the migration rate, morphology, and proliferative activity of cell demonstrated that cryogenic storage of HUC tissue for 2-3 months (and theoretically for a longer time) does not affect the efficiency of MSC isolation. As in the previous study, primary MSC cultures were derived from all collected biomaterial samples and then successfully cultured. Comparative analysis of the phenotype and multilineage differentiation potencies did not reveal significant differences between the cells isolated from fresh or frozen tissue.

Our findings confirm that considerable amount of functional MSC can be derived from frozen HUC tissue and used for creation of tissue banks and/or further development of protocols of fabrication of cell products for medical purposes.

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