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Human Umbilical Cord Mesenchymal Stromal Cell-Derived Microvesicles Express Surface Markers Identical to the Phenotype of Parental Cells

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Production of microvesicles in culture of human umbilical cord multipotent mesenchymal stromal cells was studied and comparative analysis of the expression of some surface molecules (clusters of differentiation, CD) was performed. It was found that the mesenchymal stromal cells produce microvesicles in the amount sufficient for their detection by flow cytometry. Parallel analysis of the phenotypes of maternal mesenchymal stromal cells and secreted microvesicles revealed identical expression of surface molecules CD13, CD29, CD44, CD54, CD71, CD73, CD90, CD105, CD106, and HLA-I. The concentration of microvesicles in the conditioned medium was 17.9±4.6×10^6/ml; *i.e.* one cell produced ~40-50 (44.7±11.5) microvesicles over 2 days in culture.

Key Words: *microvesicles; multipotent mesenchymal stromal cells; umbilical cord; Wharton's jelly; flow cytometry*

Multipotent mesenchymal stromal cells (MSC) and in particular MSC from umbilical cord tissue (Wharton's jelly) in recent years have been a point of interest due to their great potential in cell therapy of a wide range of acquired disorders and pathological conditions [3-8,11,20,21]. In addition to their potency to differentiate into cells of all three germ layers, umbilical cord-derived MSC are characterized by the highest proliferative and secretory activity among postnatal cells [1,4,10]. Paracrine effects of MSC are associated primarily with the production of a wide spectrum of cytokines and growth factors that can cause anti-inflammatory, anti-apoptotic, angiogenic, neuroprotective, and immunomodulatory effects under experimental and clinical conditions [1,9,12,15,23,24].

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The effects of MSC even in the absence of these cells in the lesion focus can be also mediated by microparticles, a heterogeneous group of cell-secreted structures [2,13,14,22-24]. They differ by the size, shape and mechanism of formation and can be subdivided into exosomes (40-100 nm), microvesicles (100-1000 nm), and apoptotic bodies (1-5 μ). Unlike other classes of microparticles formed by exocytosis or as a result of cell death, microvesicles (MV) are formed by external budding of the cell membrane and contain a significant amount of both internal (lipids, proteins, RNA, and microRNA) and surface molecules [14,22]. It is believed that MV can become the basis for the development of cell-free therapeutic products much safer than those containing living cells. MSC-derived MV have already demonstrated their effectiveness in the models of cardiovascular diseases, oxidative stress, ischemia/reperfusion, and in pathological conditions of the CNS, bone and cartilage tissues, lungs, and immune system [2,14,22].

Most studies of MV are based on the analysis of their purified population isolated by ultracentrifugation

at 100,000-200,000*g* or membrane filtration associated with additional methodological difficulties and the risk of damage (aggregation or degradation of particles) during isolation. Despite MV production by MSC is an established fact, little is known about MV phenotype and their quantitative characteristics.

The objective of our study was quantitative evaluation of the production of native MV by cultured human umbilical cord MSC and comparative analysis of their phenotype.

MATERIALS AND METHODS

MSC isolation and culturing. For MSC isolation, fragments of umbilical cords were obtained from healthy pregnant women at the Obstetric Departments, V. I. Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (written informed consent was signed by all participants). The technique of MSC isolation and their characteristics were described in our previous publications in detail [16-19]. Cells were cultured in DMEM/F-12 medium (Gibco) supplemented with antibiotics and 10% fetal calf serum (HyClone). After attaining confluence, the cells were the cells were passaged into new flasks using trypsin—EDTA. Passage 2-3 MSC from 3 donors were used in the study.

Preparation of the conditioned medium. Confluent MSC cultures ($5\text{-}8\times10^4$ cells/cm^2) were washed twice with Hanks balanced salt solution, a fresh portion of the culture medium was added, and the flasks were returned to CO_2 incubator. In 48 h, the conditioned media were collected, centrifuged for 20 min at 3000 rpm to precipitate cellular debris, aliquots according to the selected antibody panel, and immediately subjected to flow cytometry analysis.

The cells were washed twice with Hanks saline, harvested from plastic with trypsin—EDTA, and centrifuged for 10 min at 2000 rpm. Aliquots of cell suspensions ($2\text{-}3\times10^5$ cells in 50 μl) were used for phenotyping by flow cytometry simultaneously with the samples of the corresponding conditioned media.

Microvesicle detection. Flow cytometry (FACSCalibur, BD) was used for MV detection, counting, and phenotyping. To this end, 250 μm conditioned medium was incubated overnight at 4ºC with 20 μl of the corresponding antibodies (CD13, CD29, CD44, CD54, CD71, CD73, CD90, CD105, CD106, and HLA-I; Beckman Coulter). To evaluate the absolute number of MV, staining was carried out in TrueCount tubes (BD) containing known amount of fluorescent microspheres. For evaluation of the size of secreted particles, a calibration curve was plotted using a set of Flow Cytometry Sub-Micron size Reference Kit (Life Technologies) containing fluorescent microparticles with diameters 0.02, 0.1, 0.2, 0.5, 1, and 2 μ.

The phenotype of MSC was analyzed using the same antibody panel by the standard technique [17,18]. For simultaneous analysis of MV and MSC, the latter after incubation with the antibodies (30 min at room temperature) were washed, fixed in CellFix solution (BD), and left overnight in a refrigerator.

RESULTS

As in previous experiments, MSC used in this study expressed a wide range of surface markers (Figs. 1, 2). Almost 100% cells were positively stained for CD13, CD19, CD29, CD44, CD54 (ICAM-1), CD73, CD90, and CD105; ~50% cells expressed HLA-I. No cells positive for CD106 (VCAM-1), CD45, CD34, and HLA-DR were detected.

The pilot experiments on MV identification have demonstrated that the native culture medium used for MSC culturing contained appreciable amount of particles in the acquisition range (Fig. 3, *a*) presumably presented by protein aggregates appearing during serum freezing/thawing cycles. To exclude this artifact, the culture medium in subsequent experiments was filtered through 0.1-μ pore membrane filters. This remove unwanted noise background almost completely (Fig. 3, *b*) and allowed visualization of the MV population in parameters of side and forward light scattering (threshold values for forward/side light scatter and fluorescence were initially set at "zero"). Further gating of MV and TrueCount beads (Fig. 2, *c*-*e*) allowed us to estimate their quantity and phenotype.

Similar to maternal MSC, MV population showed positive staining for all the above listed markers (Figs. 1 and 2), but the expression level (fluorescence intensity) of some molecules (CD71, CD73, CD90, and CD105) was slightly lower than for MSC. This can be due to insufficient concentration of antibodies (in comparison with value recommended by the manufacturer) despite prolonged incubation time or with lower (in comparison with MSC) content of target antigens on MV surface. At the same time, the results for some markers (CD13, CD29, CD44, and CD54) were comparable with those obtained on MSC: almost 100% positive staining with high fluorescence intensity. Based on these findings, three markers (CD13, CD29, and CD44) were chosen for evaluation of the size and absolute content of MV in conditioned media.

By using calibration particles of known size, a linear calibration curve was plotted (Fig. 4). Unfortunately, 0.02-μ particles were below the detection sensitivity level, and 100 nm spheres were at the upper threshold of instrument sensitivity. However, most of the analyzed MV appeared to be in the range between 100 nm and 2 μ, which agree with published data [2,14,22]. Most MV are clustered in the range from 200 to 1000 μ, and

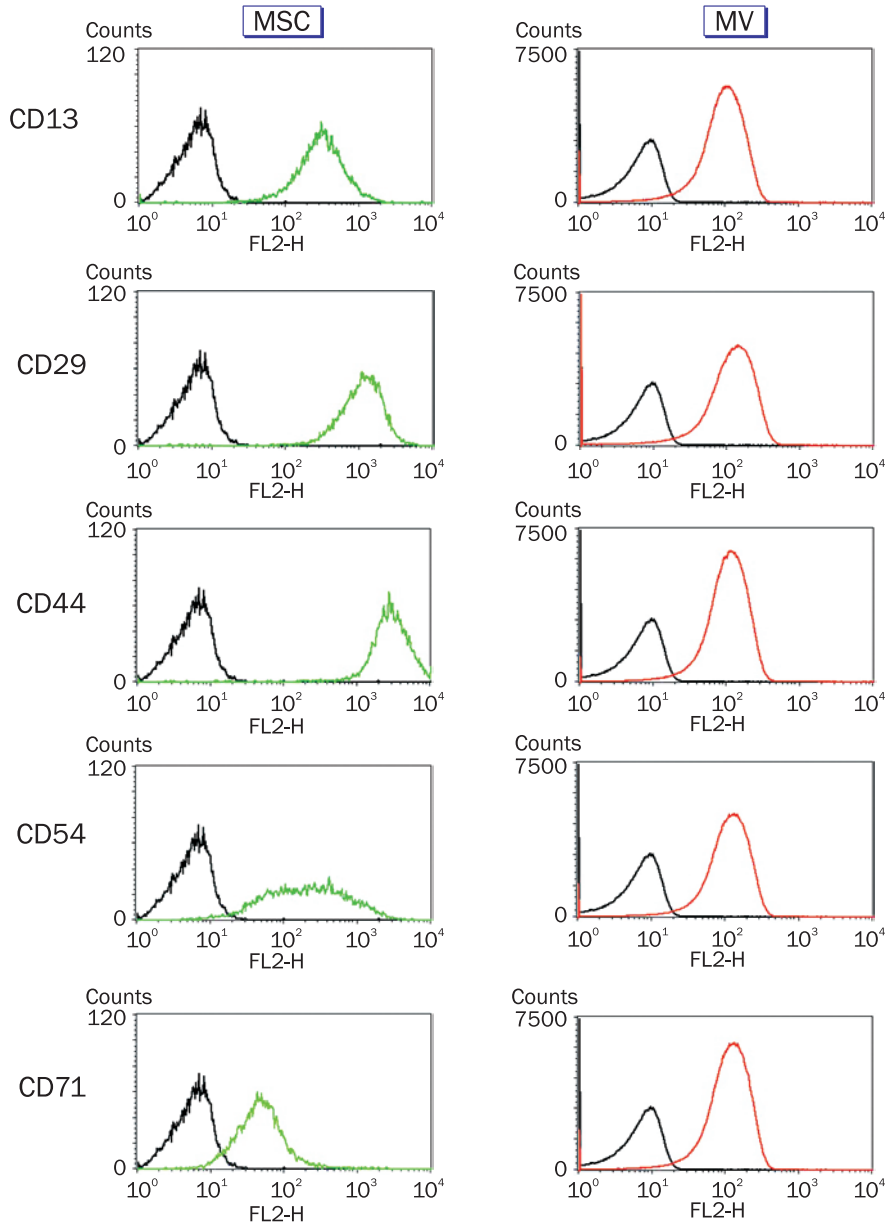

Fig. 1. Comparative analysis of the expression of surface markers CD13, CD29, CD44, CD54, and CD71 by MSC and MV.

the histogram peak was located between 300 and 500 μ, which corresponds to modern data on MV.

For evaluation of MV count and concentration, we used TrueCount tubes containing a known amount of fluorescent microparticles. This population is clearly seen in forward/side light scatter diagrams and in light scatter/fluorescence coordinates (Fig. 3, *c*, *d*). The relative and absolute number of acquired MV was calculated by the formula proposed by the manufacturer.

Unfortunately, the data on absolute values of MV production by MSC are not available in the literature, as most studies have analyzed microparticles isolated by ultracentrifugation or filtration through microfilters [2,14,22]. According to our data, the content of $CD13^+$, $CD29^+$, and $CD44^+$ MV was equal to $17.9\pm4.6\times10^6$ (min 11.6×10^6, max 22.6×10^6) particles per 1 ml medium. Thus, single MSC could produce (in the absence of additional activation) ~40-50 (44.7±11.5) MV during 48 h in culture.

Thus, MSC produce significant amounts of MV and their phenotype nearly match to a set of surface markers of parental MSC. It is noteworthy that MV fluorescence intensity in some cases was not inferior to that of MSC, which can indicate high concentration of the analyzed molecules on the membrane of these microstructures. Further studies might be aimed at more extensive analysis of synthetic/secretory activity of cells and creation of a cell-free therapeutic product.

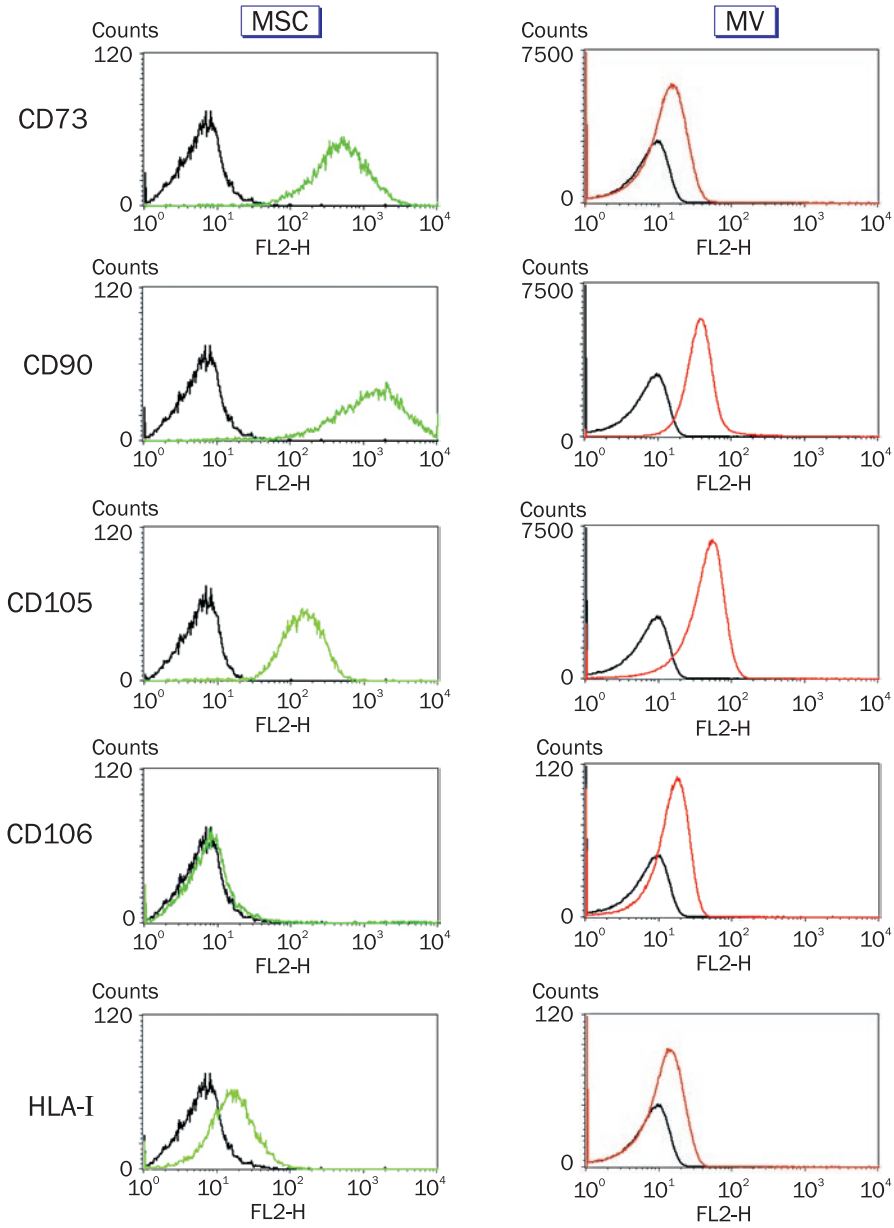

Fig. 2. Comparative analysis of the expression of surface markers CD73, CD90, CD105, CD106, and HLA-I by MSC and MV.

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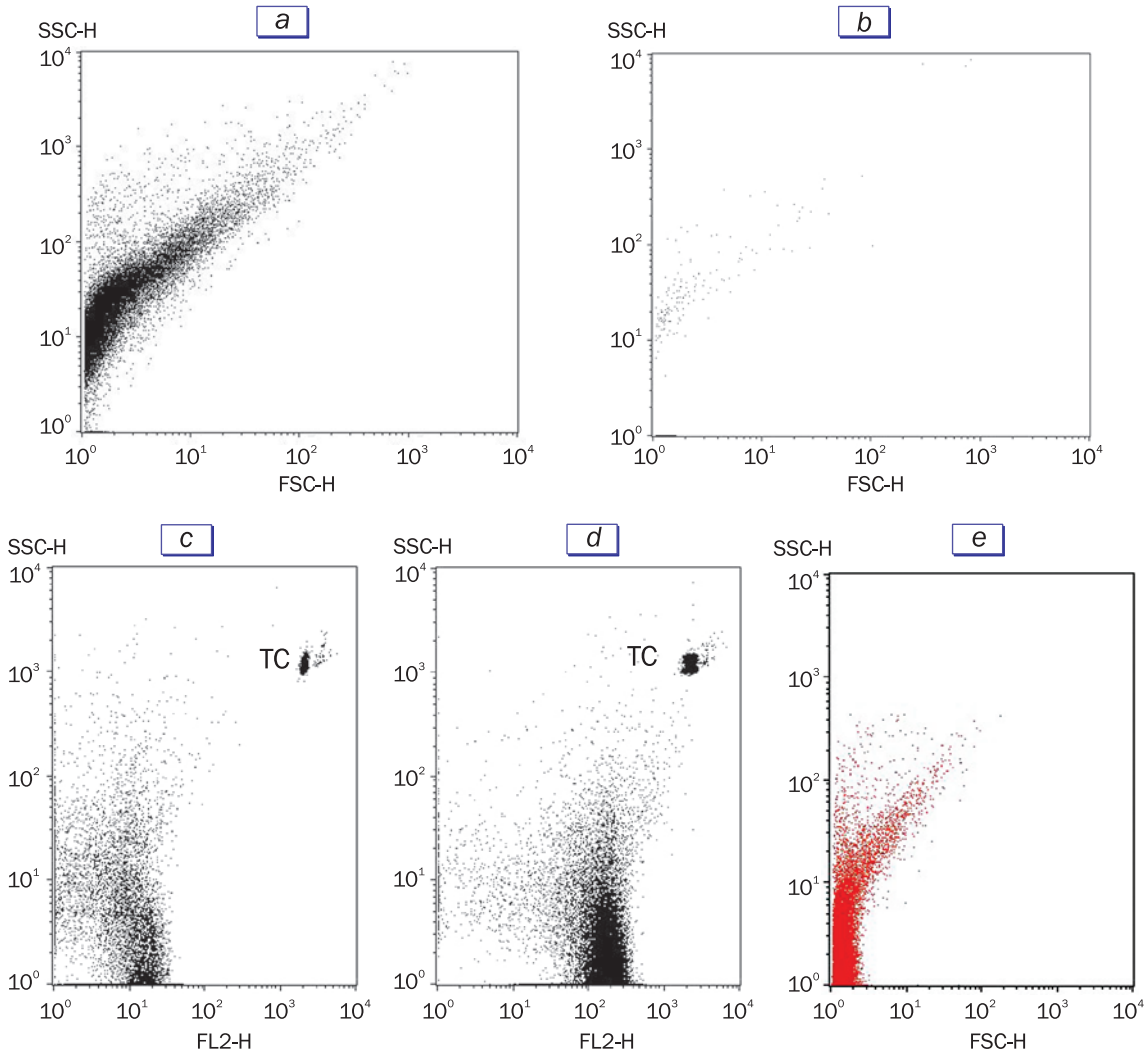

Fig. 3. A strategy of gating, counting, and phenotyping of MV by flow cytometry. *a*) Standard MSC culture medium; *b*) medium after filtration through 0.1-μ membrane filter; *c*, *d*) population of MV in MSC-conditioned medium before (isotype control) and after staining, respectively; *e*) population of positively stained MV in forward/side light scatter coordinates. TC: TrueCount beads.

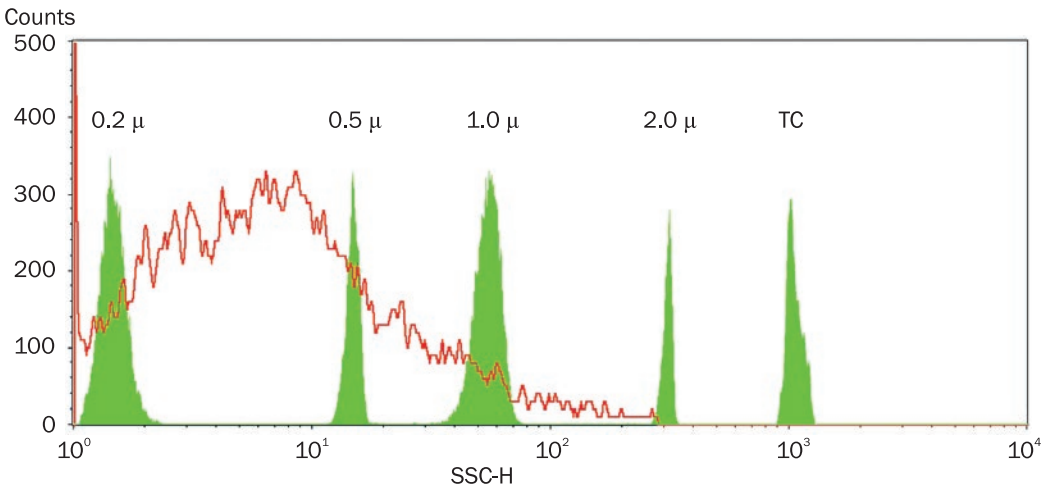

Fig. 4. Evaluation of the size of produced MV. Narrow peaks on the histogram correspond to calibration microspheres with known diameters; wide peak shows MV distribution. TC: TrueCount beads.

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