Mesenchymal Cells of the Deciduous Tooth Pulp: Cytophenotype and Initial Evaluation of Possibility of Their Use in Bone Tissue Engineering

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Cultures of mesenchymal cells from human deciduous tooth pulp were derived. The phenotype and capacity to osteogenic and adipogenic differentiation of these cells are close to those of bone marrow mesenchymal stem cells. Deciduous tooth pulp mesenchymal cells populate biodegradable polylactide scaffolds and hence, can be used for the creation of tissue engineering transplants for bone defect repair. Storage of decidual tooth pulp mesenchymal cells in the stem cell cryobanks together with umbilical blood will appreciably extent the periods of age for collection of juvenile autologous stem cells for use throughout the life span.

Key Words: *mesenchymal stem cells; tooth pulp; scaffolds; osteogenic differentiation; tissue engineering*

The development of technologies for bone defect repair is a priority trend of tissue engineering. One of the prospective approaches in this sphere is population of biodegradable matrices (scaffolds) by cells with osteogenic potential under *ex vivo* conditions with subsequent transplantation into the bone defect zone. Autologous mesenchymal stem cells (MSC) isolated from the bone marrow (BM) [4] or adipose tissue [5] and reproduced in culture are most often used as cell material for the scaffold population. Unfortunately, MSC, similarly as all other cells in human body, are aging throughout life [2] and are exposed to unfavorable environmental factors, which leads to accumulation of somatic mutations and reduction of the proliferative and osteogenic potential [3]. An effective approach to the solution of this problem is the use of osteogenic cells from autologous deciduous tooth pulp. After isolation and expansion in culture, these cells can be stored in liquid nitrogen in the cryobank until use.

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We obtained cultures of human deciduous tooth pulp mesenchymal cells (MC) and BM MSC and compared their cytophenotypical characteristics and capacity to adipogenic and osteogenic differentiation and population of polylactoglycolide-based biodegradable scaffolds.

MATERIALS AND METHODS

Isolation and culturing of cells. Bone marrow MSC were isolated from aspirated human BM. Mononuclear cells were isolated in Ficoll-urograffin gradient (PanEco) and cultured in growth medium (DMEM/F12; 1:1) with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (all reagents from Gibco) until monolayer formation. The medium was replaced twice a week.

Deciduous teeth were collected after their natural exfoliation. The material was washed in Hanks' solution with cultural antibiotic/antimycotic (Gibco). After opening the crown, the pulp was extracted, fragmented, and incubated in 0.1% collagenase-1 (Gibco) at 37°C for 90 min. The resultant cells were precipitated

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by centrifugation, resuspended in growth medium, and cultured until the formation of a monolayer. The medium was replaced twice a week.

Flow cytofluorometry. Cytophenotyping of the resultant primary cultures was carried out by flow cytofluorometry. Monoclonal antibodies to surface antigens conjugated with FITC and phycoerythrin (Catlag) were used. The cells were suspended in versen solution and incubated in PSB with 1% fetal calf serum with monoclonal antibodies in the working concentrations, after which were fixed in 4% paraformaldehyde. The study was carried out on a FACSAria flow sorter cytofluorometer (Becton Dickinson). The data were processed using WindMDI software.

Adipogenic differentiation of cells in culture. Decidual tooth pulp MC and BM MSC were cultured on slides. Adipogenic differentiation of the cells of the studied cultures was carried out in serum-free growth medium with 10% horse serum, 0.5 mM isobutylmethylxanthine, and 60 μM indomethacin for 7 days, after which the preparations were fixed in 4% paraformaldehyde and stained with Oil Red.

Osteogenic differentiation of cells in culture. The cells were cultured on slides. Decidual tooth pulp MC and BM MSC differentiated into bone tissue in serum-free medium with 0.2 mM ascorbic acid, 10 mM calcium β-glycerophosphate, and 10^{-7} M dexamethasone (all reagents from Sigma). The cells were cultured for 3 weeks, the medium was replaced twice a week.

Osteogenic differentiation of the cells was evaluated on days 8, 15, and 22 of culturing. The preparations were fixed in 4% paraformaldehyde and stained with Alizarin Red. In addition, changes in the level of osteonectin expression and endogenous alkaline phosphatase activity were evaluated on day 8. Osteonectin expression was studied by immunohistochemical staining with primary monoclonal antibodies to human osteonectin and second rhodamine-conjugated antispecies antibodies. The nuclei were poststained with DAPI.

Immunohistochemical reaction to alkaline phosphatase was carried out by plunging cell preparations on slides into reaction mixture containing 100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 5 mM $MgCl_2$, 1 mg/ml nitroblue tetrazoleum, and 0.1 mg/ml BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) (all reagents from Sigma) with subsequent 30-min incubation at 37°C. The reaction was stopped by adding 10 mM EDTA.

Population of 3D polylactide matrices with cells. Cells of the studied cultures were dripped in a 200-μl (1 mln/ml concentration) droplet onto scaffolds (5×5×1 mm plates) under sterile conditions. 3D biodegradable polylactoglycolide-based scaffolds with pores of two orders with the mean diameters of 30 and 600 μ were obtained by surface selective laser sintering at Institute for Problems of Laser and Information Technologies After 60-min incubation at 37°C needed for effective adhesion of cells, the populated scaffolds were plunged into the growth medium. Subsequent culturing was carried out in a CO_2 incubator (5% CO_2) at 37°C; the medium was replaced twice a week.

Microscopy. The preparations were examined under an AxioPlan 2 fluorescent microscope (Carl Zeiss). The results were recorded using an AxioCam HRc digital camera.

RESULTS

Primary adherent cultures of human deciduous tooth pulp MC and BM MSC were obtained. Cells of both cultures had a fibroblast-like morphology characteristic of MC.

The cell cultures were tested for expression of stem and progenitor cell surface marker proteins CD29, CD34, CD44, CD45, CD49b, CD54, CD90, CD105, CD106 and HLA-DR (Table 1).

No expression of hemopoietic stem cell markers CD34, CD45, and HLA-DR was detected.

CD44, CD54, CD90, CD105, and CD106 are surface proteins characteristic of multipotent mesenchymal cells. They play an important role in cell-cell interactions, cell migration, homing, and differentiation. Cells of both studied cultures exhibited intensive expression of CD44, CD105, and CD90 and lower expression of CD54 and CD106.

CD49b and CD29 marker molecules are, respectively, α_2- and β_1-subunits of VLA-2 integrin binding

TABLE 1. Cytophenotypical Profiles of BM MSC and Deciduous Tooth Pulp MC Cultures

Marker	BM MSC	Deciduous tooth pulp MC
CD34	–	–
CD45	–	–
HLA-DR	–	–
CD29	±	+
CD44	+	+
CD49b	–	+
CD54	±	±
CD90	+	+
CD105	+	+
CD106	±	±

Note. «+»: high expression; «±» low expression; «–»: no expression.

to collagen and laminin. This peptide complex provides cell interaction with extracellular matrix [1]. The expression of these markers in the tooth pulp cell culture was higher than in BM MSC, presumably due to higher collagen content in the extracellular matrix of deciduous tooth pulp in comparison with BM.

Hence, the immunocytochemical study showed that the cell phenotype of the cultures corresponded to progenitor mesenchymal cells [8]. No appreciable differences in the phenotypical profiles of deciduous tooth pulp MC and BM MSC were detected.

The capacity of deciduous tooth pulp MC and BM MSC cultures to differentiation into adipose and bone tissues was evaluated.

Adipogenic differentiation was induced by adding equine serum, isobutylmethylxanthine, and indomethacin into culture medium. After 7 days of culturing in medium for adipogenic differentiation, all cells of the studied cultures accumulated lipid vacuoles specifically stained with Oil Red.

The cells differentiated into osteoblasts in serum-free medium with ascorbic acid, sodium β-glycerophosphate, and dexamethasone. Changes in the expression of osteonectin and activity of alkaline phosphatase and accumulation of calcium salts during differentiation were evaluated.

Osteonectin is one of the main proteins of the bone tissue matrix. It is a calcium-binding glycoprotein with a molecular weight of 43 kDa. At the initial stages of osteogenic differentiation the expression of this marker in tooth pulp and BM MSC cultures remained basal. By day 8, the expression of osteonectin increased in both cultures.

Alkaline phosphatase catalyzes hydrolysis of phosphoric acid complex esters. This enzyme plays an important role in bone formation and growth. At the initial stage just solitary cells with low activity of alkaline phosphatase were detected in BM MSC culture; no activity of the enzyme was detected in tooth pulp cell culture (Fig. 1, *a*, *b*). By day 8 of culturing in medium for osteogenic differentiation, activity of

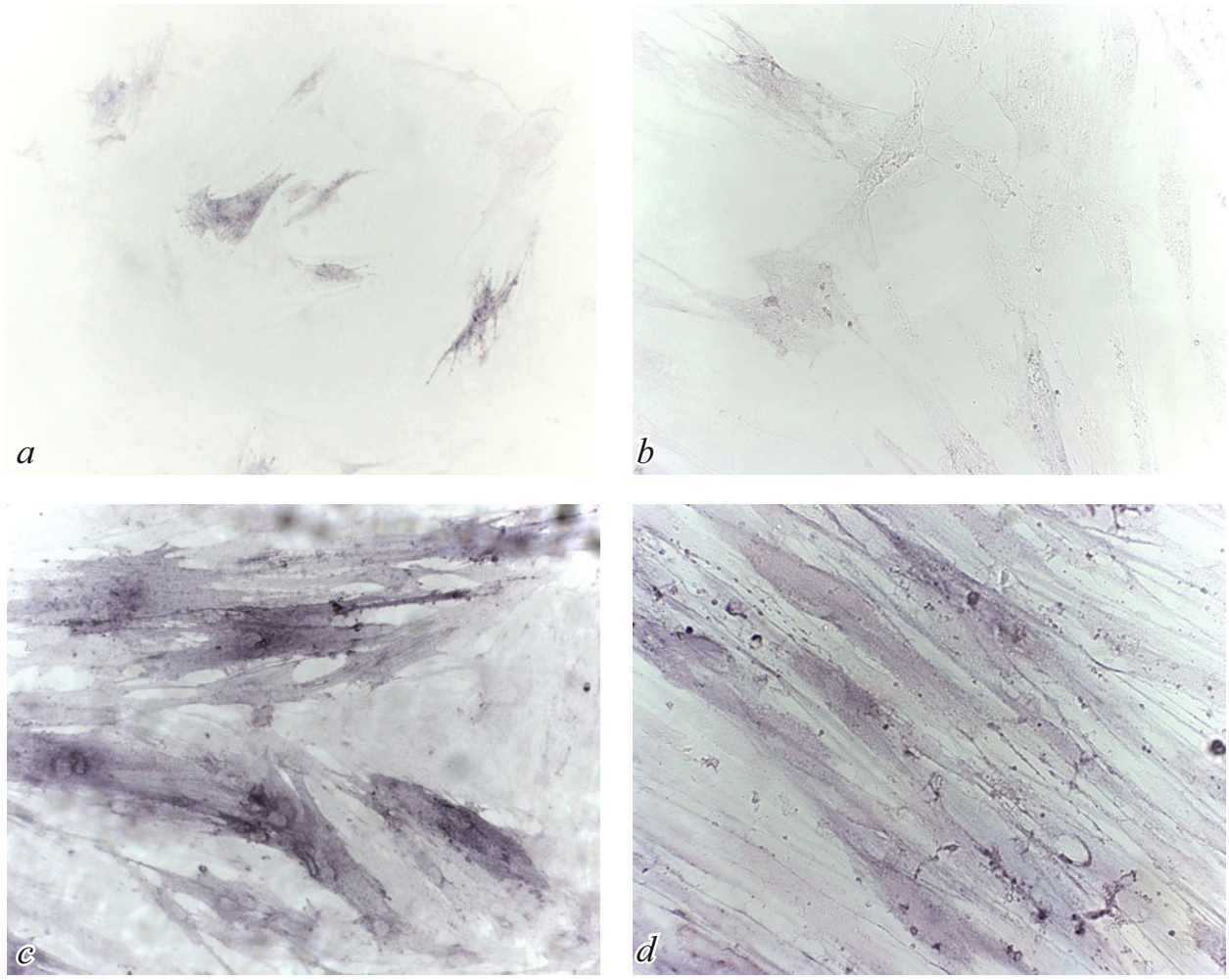

Fig. 1. Activity of alkaline phosphatase in BM MSC (*a*, *c*) and deciduous tooth pulp MC (*b*, *d*) on days 1 (*a*, *b*) and 8 (*c*, *d*) of osteogenic differentiation (×630).

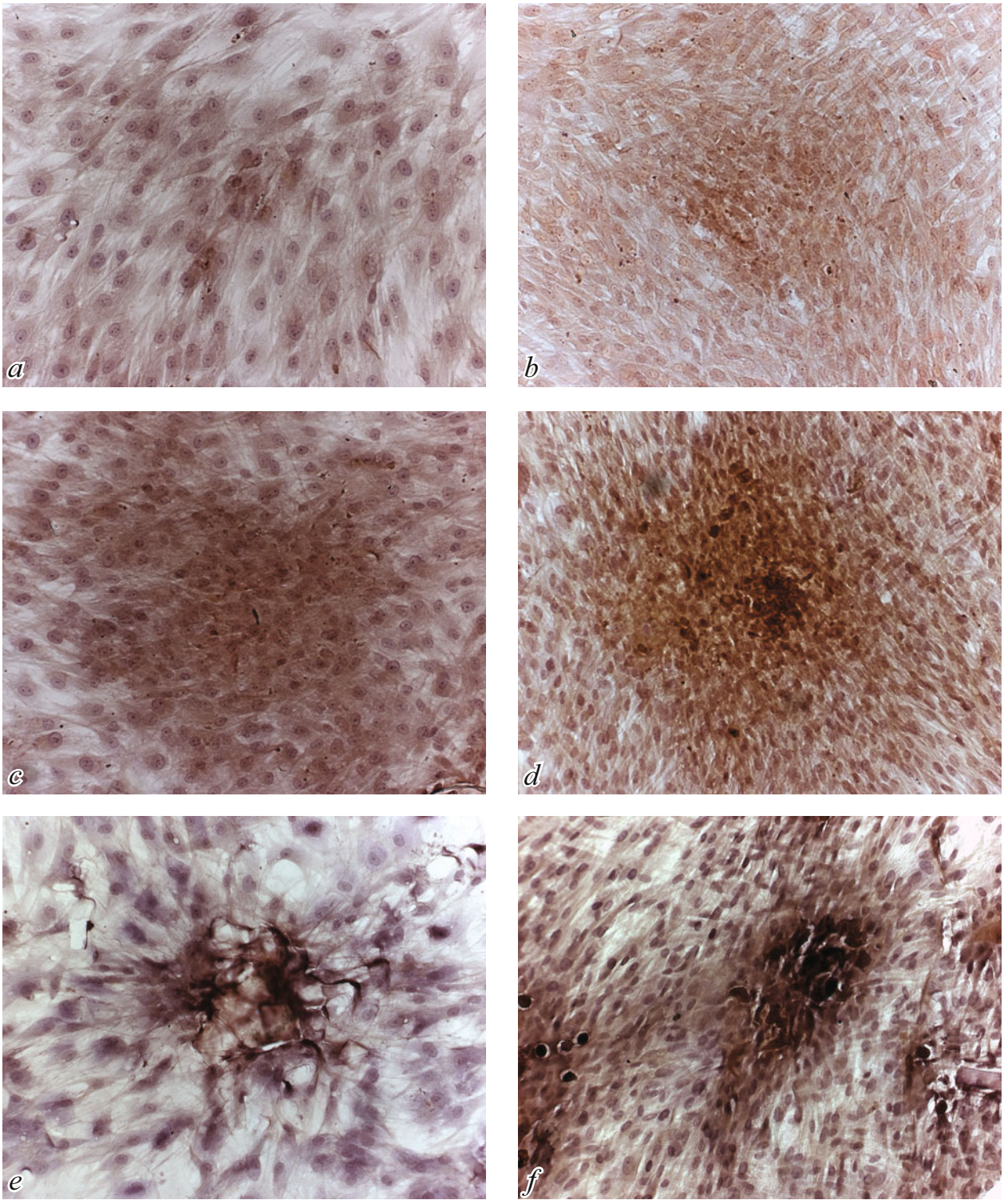

Fig. 2. Accumulation of calcium phosphates by BM MSC (*a*, *c*, *e*) and deciduous tooth pulp MC (*b*, *d*, *f*) on days 8 (*a*, *b*), 15 (*c*, *d*), and 22 (*e*, *f*) of osteogenic differentiation. Alizarin Red staining (×200).

alkaline phosphatase critically increased in all cells of the studied cultures (Fig. 1, *c*, *d*).

Accumulation of calcium salts by the studied cells during differentiation into osteoblasts was detected by Alizarin Red staining. Areas of high concentration of cells specifically binding the stain were detected on day 8 of culturing in medium for osteogenic differentiation (Fig. 2, *a*, *b*). Later, the cell density in these foci increased. The cells in these foci actively accumulated calcium phosphates, which manifested by

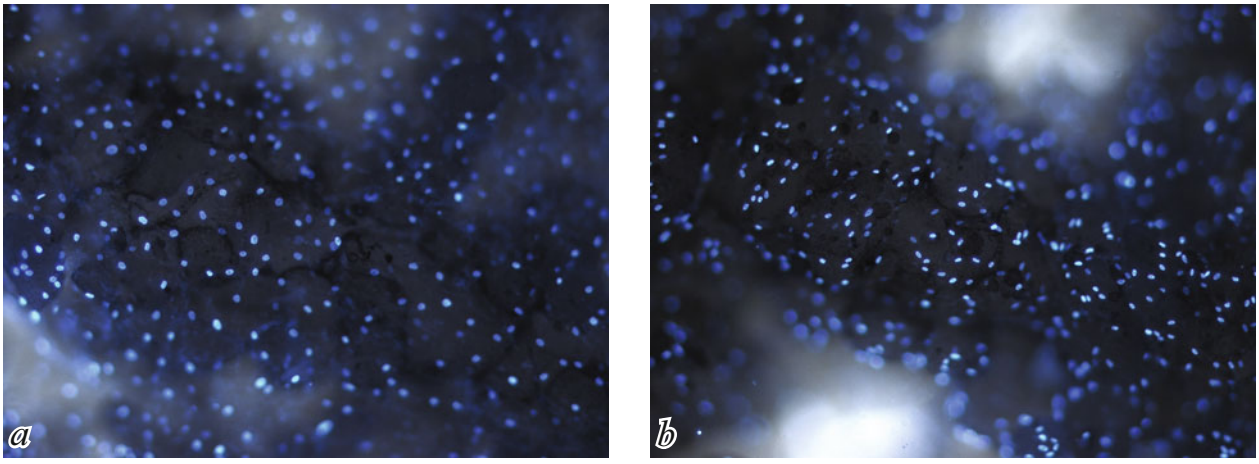

Fig. 3. Bone marrow MSC (*a*) and deciduous tooth pulp MC (*b*) cultured on synthetic biodegradable matrix. Nuclei stained with DAPI (×100).

more intense staining by day 15 of culturing (Fig. 2, *c*, *d*). By day 22, an appreciable number of ossification foci characterized by the presence of cells with the morphology typical of osteoblasts and high content of calcium salts in vacuoles was seen in both cultures (Fig. 2, *e*, *f*). The adjacent cells were actively involved in osteogenic differentiation.

The results of this study indicate that human deciduous tooth pulp fibroblast-like cells are characterized by osteogenic potential similar to that of human BM MSC.

In order to confirm the conclusion on good prospects of using human deciduous tooth pulp MC as material for creation of tissue engineering constructions, we attempted population of 3D biodegradable polylactoglycolide-based scaffolds by the studied cells. Appreciable numbers of deciduous tooth pulp MC and of BM MSC were detected on day 8 after application of these cells to scaffolds. Viability of cells was retained after 4 weeks of culturing in both cases; no apparent difference between the two cell cultures was detected (Fig. 3).

Our results indicate that deciduous tooth pulp MC and BM MSC have similar cytophenotypical profiles and almost identical capacities to adipogenic and osteogenic differentiation and to homing in scaffolds *in vitro*. Hence, the deciduous tooth pulp is a prospective source of cell material for bone tissue engineering. In addition, deciduous tooth pulp MC can presumably be used for creation of regeneration technologies for other types of connective tissue, for example, cartilage, tendons, and ligaments. It is known that MC cultures derived from deciduous tooth pulp contain pluripotent cells capable of neurogenic, myogenic, and hepatogenic differentiation. These cells were called stem cells from human exfoliated deciduous teeth (SHED) [6,7]. Hence, the deciduous tooth pulp MC can be stored at banks of autologous stem cells. At present, only umbilical blood specimens are stored in these banks. However, the counts of pluripotent cells in umbilical blood vary and are sometimes insufficient for therapeutic use. Hundreds of millions of MC can be obtained from exfoliated deciduous tooth pulp. In addition, the number of children whose autologous stem cells can be stored for subsequent use throughout life is appreciably increased.

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