Design of Tissue Engineering Implants for Bone Tissue Regeneration of the Basis of New Generation Polylactoglycolide Scaffolds and Multipotent Mesenchymal Stem Cells from Human Exfoliated Deciduous Teeth (SHED Cells)

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Cultures of multipotent mesenchymal stromal cells from the pulp of human deciduous teeth (SHED cells) were characterized. The cells were used for population of 3D biodegradable polylactoglycolide scaffolds; their osteogenic potential was preserved under these conditions. Implantation of the scaffolds to mice induced no negative reactions in the recipients. These results suggest that the use of polylactoglycolide scaffolds populated with SHED cells is a promising approach for creation of implants for bone defect replacement.

Key Words: *mesenchymal stem cells; dental pulp; biodegradable scaffold; selective laser sintering; tissue engineering*

Tissue engineering of the bone implies the use of osteogenic cells in a complex with a scaffold. Scaffold immobilizes cells on its surface, ensures mechanical strength of the construct, and imitates bone tissue matrix that normally interacts with osteoprogenitor cells and mediating realization of their functions.

The use of biodegradable scaffolds for bone tissue engineering makes possible complete replacement of the implant with regenerating bone tissue. In parallel, the boundary between the bone tissue and the implant disappears and mechanical load in this area decreases. Polylactoglycolide, a co-polymer of lactic and glycolic acids, is one of the most promising materials for creation of bone implants. The technology of creation of polylactoglycolide scaffolds allows modification of their characteristics affecting cell adhesion and invasion [6] and the rate of resorption [3].

The aim of our study was isolation of cultures of multipotent mesenchymal stromal cells from the pulp of human exfoliated deciduous teeth (SHED cells), their cytophenotypic characteristics, population of new-generation biodegradable polylactoglycolide scaffolds with these cells, evaluation of their osteogenic differentiation capacity in the prepared tissue engineering complexes, and preliminary evaluation of the possibility of using the prepared scaffolds as bone implants in *in vivo* experiments.

MATERIALS AND METHODS

Cell isolation and culturing. Naturally exfoliated deciduous teeth were collected for the study. The material was washed in Hanks solution with antibiotic-antimycotic for cell cultures (Gibco). After crown opening, the pulp was removed, minced, and incubated in 0.1%

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type 1 collagenase (Gibco) at 37°C for 90 min. The cells were precipitated by centrifugation, resuspended in growth medium (DMEM/F12, 1:1) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (all reagents from Gibco), and cultured until confluence. The medium was changed 2 times a week.

Flow cytofluorometry. The obtained primary cultures were cytophenotyped by flow cytometry. We used monoclonal antibodies to surface antigens conjugated with FITC, allophycocyanin (APC), or phycoerythrin (PE; Becton Dickinson). The cells were suspended with versen solution (PanEko), incubated in PBS supplemented with 1% fetal calf serum and containing monoclonal antibodies in working concentration, and then fixed with 4% paraformaldehyde. Analysis was performed on FACSAria cytofuorometer sorter (Becton Dickinson). The results were processed using WinMDI software.

Preparation of scaffolds. Synthetic biodegradable 3D polylactoglycolide scaffolds were fabricated by the method of surface-selective laser sintering on a SLS-100 device developed at Institute of Laser and Information Technologies, Russian Academy of Sciences [3]. Single-mode fiber laser with 1.06 μ wavelength and 10 W power (IRE-Polus) served as continuous radiation source. The scanning rate was 21 cm/sec and the diameter of laser beam was 125 μ.

Particles of bioresorbed polylactoglycolide polymer Purasorb 7507 (Purac) with characteristic viscosity of 0.63 dl/g and mean diameter of 200 μ were subjected to sintering. Carbon nanoparticles were added to the polymer powder as the main adsorbent of laser radiation (0.1 wt%). The specific geometric surface of the absorber was 50 m^2/g.

The scaffolds were sterilized by plunging into 70% ethanol followed by 24-h incubation in Hanks solution (PanEko) with antibiotic-antimycotic for cell cultures (Gibco).

Population of the scaffolds with cells. The cells were suspended by treatment with 0.25% trypsin with versene (1:1, PanEko), precipitated, and applied in drops (200 μl, cell concentration 10^6/ml) on disk-shaped scaffolds (10×1 mm) placed in wells of a 24-h well plate. The plate containing scaffolds with applied cells was incubated for 45 min in a CO_2 incubator under standard conditions providing effective adhesion of cells. Then, growth medium was added to wells.

Osteogenic differentiation of cells in culture. Differentiation of the obtained cultured into bone tissue was performed in a serum-free medium containing 0.2 mM ascorbic acid, 10 mM calcium β-glycerophosphate, and 10^{-7} M dexamethasone (all reagents are from Sigma). The cells were cultured for 2 weeks. The medium was changed 2 times a week.

Immunocytochemical staining. Cell cultures were fixed with paraformaldehyde and permeabilized with 1% Triton X-100 (Sigma); nonspecific binding of antibodies was blocked by incubation in PBS containing 0.5% BSA, 2% normal goat serum, 0.05% Tween 20, and 0.01% merthiolate (Sigma). The cells were incubated with primary monoclonal antibodies to osteocalcin (Chemicon), washed, and incubated with interspecies antibodies conjugated with fluorescent dye DyLight 488 (R&D Systems). The nuclei were

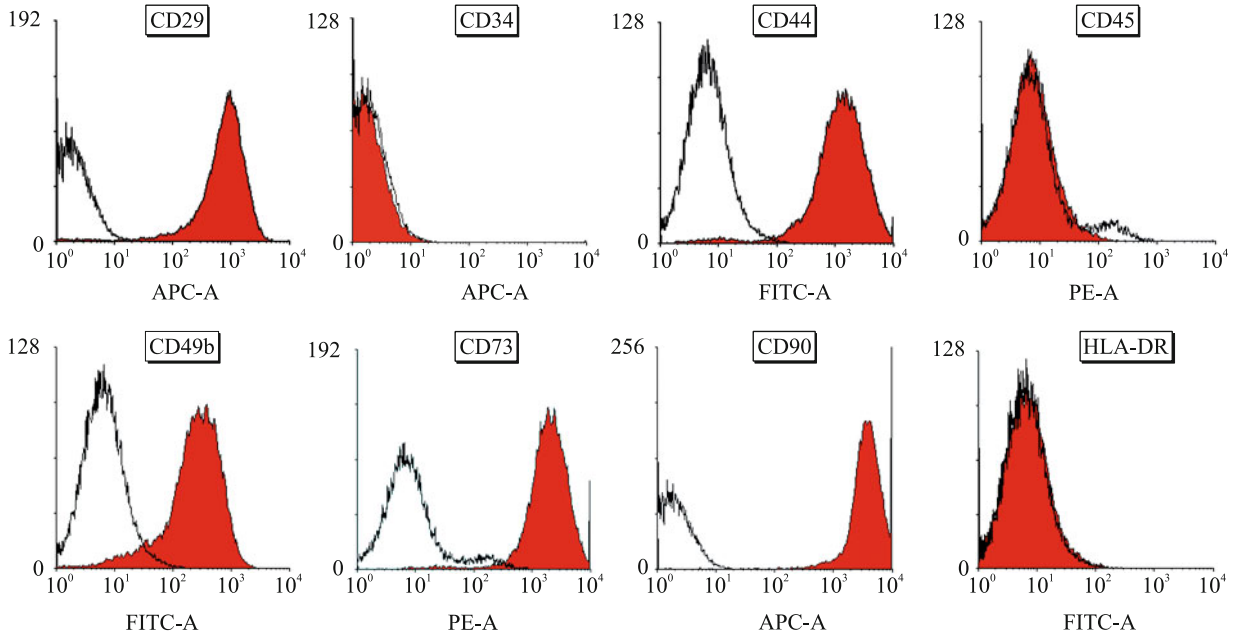

Fig. 1. Expression of CD29, CD34, CD44, CD45, CD49b, CD73, CD90, and HLA-DR in culture of SHED cells. Abscissa: fluorescence intensity reflective the level of marker expression; ordinate: number of events (cells). Black line: isotypic control.

poststained with DAPI (Sigma). Cells within tissue engineering complexes and cells seeded on coverslips after culturing in scaffolds were stained.

In vivo experiments. Scaffolds of cylindrical shape (5×5 mm) were implanted subcutaneously to BALB/c mice. After 1 month the animals were sacrificed and the implants were removed and analyzed. Longitudinal 1-mm sections of isolated scaffolds were fixed with paraformaldehyde and stained with DAPI for detection of cells.

Microscopy. Microscopy was performed using an AxioPlan 2 microscope (Zeiss). The results were photographed using an AxioCam HRc digital camera.

RESULTS

Adherent fibroblast-like cell cultures were isolated from the pulp of exfoliated deciduous teeth. Passage 3 cells were tested for expression of CD29, CD34, CD44, CD45, CD49b, CD73, CD90, and HLA-DR (Fig. 1).

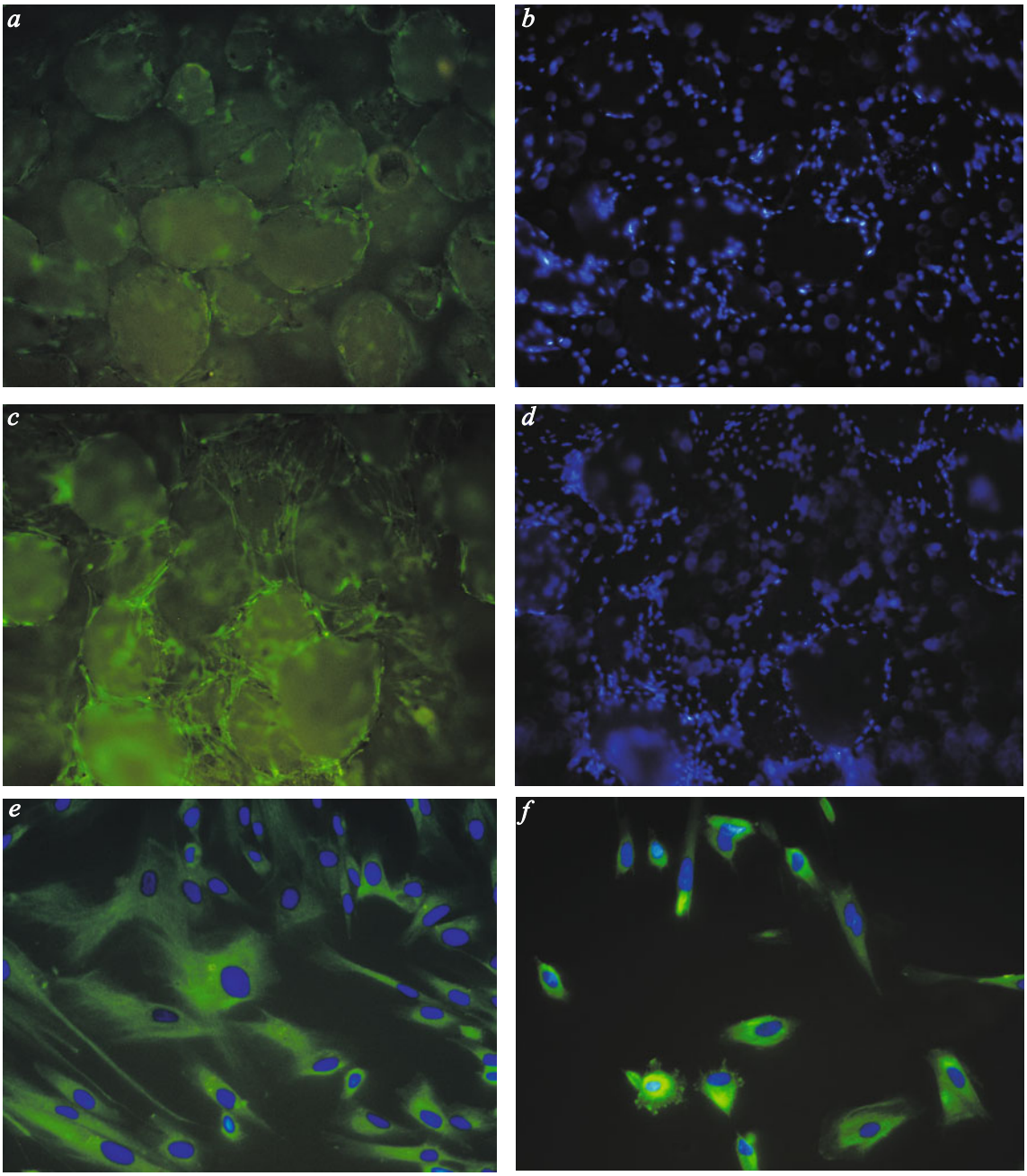

Fig. 2. Expression of osteocalcin in SHED cells cultured on polylactoglycolide scaffolds. *a, b, e*: cells cultured in growth medium; *c, d, f*: cells cultured in osteogenic differentiation medium. *a-d*: immunocytochemical staining of cells of scaffolds (*a*,*c*: staining with antibodies; *b*,*d*: staining of nuclei with DAPI); *e*,*f*: immunocytochemical staining on coverslips (superposition). Magnification: ×100 (*a-d*); ×400 (*e, f*).

Expression of hemopoietic SC markers CD34, CD45, and HLA-DR was not detected in the test cultures (Fig. 1, *a*), whereas surface molecules CD44, CD73, and CD90 typical of stem and progenitor mesenchymal cells were clearly expressed (Fig. 1, *b*). On the whole, the isolated cells phenotypically corresponded to multipotent mesenchymal stromal cells [5]. Moreover, high expression of CD49b and CD29, subunits of integrin VLA-2, was observed in the test cultures (Fig. 1, *c*), which according to published data was typical of SHED cells [2].

Then, polylactoglycolide scaffolds were populated by isolated SGED cells and osteogenic differentiation of these cells was induced. To this end, tissue engineering complexes were cultured in serum-free medium supplemented with ascorbic acid, calcium β-glycerophosphate, and dexamethasone. In 14 days of differentiation, changes in osteocalcin expression in cells were evaluated by immunocytochemical staining.

Osteocalcin (BGLAP, bone gamma-carboxyglutamic acid-containing protein) is the main non-collagen protein of the bone participating in binding of calcium and hydroxyapatite. The main function of this protein is participation in the formation of bone matrix. Active center of the protein molecule contains a negatively charged site binding Ca^{2+} ions. Upon binding with hydroxyapatite, osteocalcin releases calcium, thus promoting crystal growth [8]. Due to this, osteocalcin ensures regular organization of the mineral component of the forming bone matrix. Osteocalcin is known to be a chemoattractant for mesenchymal cells [14] and angiogenesis inductor [4], which suggests that this protein participated in regeneration of the bone tissue. Osteocalcin is used as a marker of osteogenic differentiation [7].

According to our data, osteocalcin is more intensively expressed in SHED cell culture on scaffolds in a medium containing inductors of osteogenic differentiation than during culturing in a simple growth medium (Fig. 2). Hence, the cells specifically responded to induction of osteogenic differentiation, which indicates their osteogenic potential and the absence of negative effects of the scaffold on viability and differentiation of cultured cells.

In parallel with evaluation of the effect of scaffold material on osteogenic potential of SHED cells *in vitro* we studied the behavior of polylactoglycolide scaffolds in experiments *in vivo*. To this end, the samples were subcutaneously implanted to mice for 1 month and then removed and analyzed. Visually, no signs of inflammatory reaction were found at the site of implantation; the implants were coated with a capsule containing considerable number of blood vessels (Fig. 3, *a*). On section (Fig. 3, *b*), active migration of recipient cells from the capsule into the scaffold was observed: invasion is possible due to porous structure of the scaffold. Thus, the results of preliminary evaluation suggest that the studied scaffolds are a promising material for creation of tissue-engineering implants for bone tissue regeneration.

Resent studied in the field of bone implants are devoted to the problem of improving osteoinductive properties of scaffold fabricated from polylactide copolymerized with polyglycolide by addition of components stimulating cell migration, proliferation, and differentiation. Growth factors [9,11] and nanosized particles of hydroxyapatite [10], bioactive glass [13], and titanium [12] are most often used for this purpose. In light of this, the possibility of incorporation of osteoinductive substances into scaffold material is an important characteristic of any technology. Due to the use of above-critical fluid technology, calcium phosphate nanoparticles (hydroxyapatite, carbonate-substituted hydroxyapatite, octacalcium phosphate, *etc.*) can be incorporated into the developed scaffolds [1].

The pulp of deciduous teeth is a promising source of cell material for scaffold population in tissue bone engineering. Our experiments and published data sug-

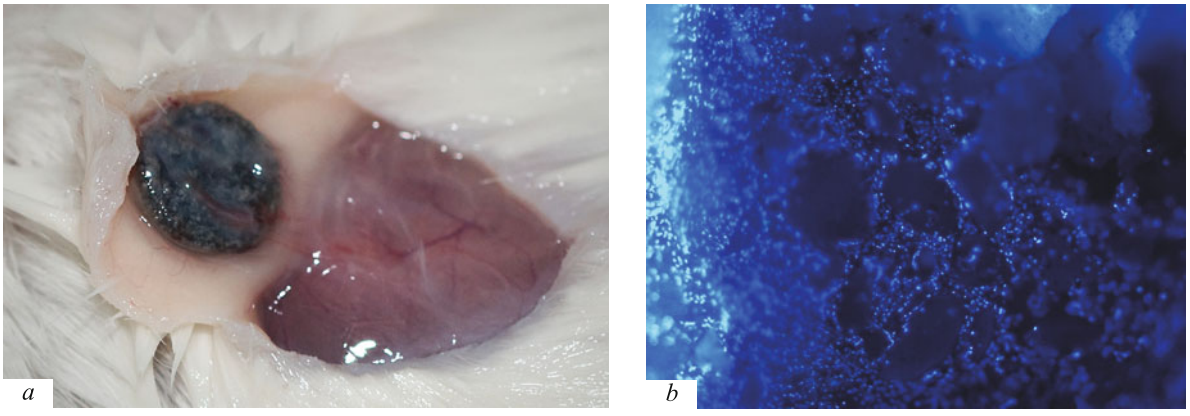

Fig. 3. Polylactoglycolide scaffold implanted subcutaneously to a mice for 1 month (×100). *a*) general appearance; *b*) invasion of recipient cells into the scaffold (left to right). Nuclei are stained with DAPI.

gest that considerable amounts of multipotent mesenchymal cells with high osteogenic differentiation potential can be isolated from exfoliated deciduous tooth. Moreover, SHED cells can be easily obtained, because the material can be easily and non-invasively collected and the cells are characterized by high proliferative activity, which makes them an attractive material for storing in cryobanks of autologous SC. On the other hand, polylactoglycolides are a perspective material for scaffold fabrication.

Thus, the above approaches can be successfully used for bone tissue engineering.

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