Immobilization and Long-Term Culturing of Mouse Embryonic Stem Cells in Collagen-Chitosan Gel Matrix

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We propose a method of creation of a 3D matrix consisting of native collagen fibers and natural polysaccharide chitosan. The collagen-chitosan hydrogels maintain viability and prolipherative activity of embryonic stem cells obtained from internal cells of mouse blastocyst. The proposed system forming hydrogels *in situ* can be used in cell therapy for immobilization and targeted delivery of stem cells.

Key Words: *collagen; chitosan; hydrogel; stem cells*

The organism responds to injuries by intensive cell proliferation and activation of synthesis of collagen and polysaccharides filling the tissue defect. Precursor stem cells migrate within the collagen-polysaccharide gel, proliferate, differentiate, and restore normal tissue morphology. The composition and structure of the microenvironment determine the rate of proliferation and type of differentiation of stem cells and, finally, the rate and degree of tissue regeneration. The study of the effect of microenvironment on the growth and development of multipotent cells is important for the use of embryonic stem cells (ESC) in cell therapy, when the creation of microenvironment regulating cell differentiation in a certain direction and preventing malignant transformation of the tissue is an urgent problem.

Collagen gels are a matrix providing normal functioning of most cell types and stimulating regeneration processes. Collagen gels embedding immobilized cells and bioactive factors are successfully used in clinical practice. However, this system cannot produce long-term positive effects on tissue regeneration because of high rate of collagen biodegradation. Moreover, high content of collagen can induce fibroid differentiation of polypotent stem cells and cicatrix formation.

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Chitosan (poly(1-4)2amino-2-deoxy-D-glucose) is a deacetylated derivative of widespread natural biopolymer chitin. It is characterized by higher resistance to biodegradation compared to collagen. This polysaccharide can absorb 2-5 water molecules per 1 monomer moiety and its biodegradation products (N-acetylglucosamine or glucosamine) are non-toxic, non-immunogenic, and can be utilized in the organism for glycoprotein synthesis. It is known that chitosan derivatives are immunologic adjuvants and polyclonal B-cell activators; they reduce the risk of tumor transformation of tissues. Administration of chitosan can considerably improve the safety of stem cell application in medicine [6]. Chitosan is more resistant to biodegradation than collagen and chitosan gels can provide chondrocyte immobilization and restoration of the cartilage tissue [1]. The absence of adhesion sites does not support the survival of substrate-dependent cells in the chitosan gel matrix, but this can be a positive factor for the maintenance of stem cell potency.

Collagen-chitosan mixtures are not available in nature, but their specific properties allow to create materials with unique structural and mechanical characteristics and high biocompatibility [2,4-6]. The possibility of varying protein and polysaccharide components allowed adaptation of physicochemical characteristics of this system for the growth of substrate-dependent cells (fibroblasts [4], epi-

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thelial cells [5]). These materials are porous sponges or films obtained by lyophilization or drying of collagen-chitosan solutions and stabilized with fixers (*e.g.* glutaraldehyde).

The aim of the present study was to develop injectable hydrogels capable of forming 3D matrix *in situ* and intended for immobilization and targeted delivery of stem cells.

We developed a procedure for the formation of a combined hydrogel preserving stability of chitosan gel and not disturbing the structure of collagen fibers and studied the possibility of culturing ESC within the collagen-chitosan hydrogel.

MATERIALS AND METHODS

The study was performed on (CBA×C57Bl)F_1 mice. For obtaining ESC, stimulation of superovulation was performed in 2-3-month-old mice. To this end, the mice received subcutaneously 10 U pregnant mare's serum gonadotropin (Follimag) and after 48 h they intraperitoneally received 15 U human chorionic gonadotropin. The embryos were isolated on day 4 of pregnancy at the stage of morula and early blastocyst. The embryos were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS to the hatched blastocyst stage (Fig. 1). The embryos adhered to the cultural flask bottom and formed trophectodermal processes with clear-cut discernible node containing internal mass cells (IMC) on their surface. Separation of IMC node formed on the top of the trophectodermal layer was performed on day 1-7 of culturing in DMEM supplemented with 20% FCS and leukemia-inhibiting factor (10 ng/ml) using mechanical detachment with a pipette (Fig. 2).

Human embryonic fibroblasts were obtained by enzyme disaggregation of embryonic tissues (8-10 weeks): aseptically isolated tissues were washed with DMEM, minced, and 0.5×0.5-mm fragments were treated with 0.25% trypsin at 37°C for 15-20 min. The isolated cells were centrifuged at 1500 rpm for 2 min and cultured in DMEM supplemented with 10% FCS. Fractionation of the primary mixed culture was carried out using differences in adhesion time for different cell types. For preparing feeder layer, the cell monolayer was treated with mitomycin C (DMEM, 10% FCS, 1 μg/ml mitomycin C) for 3 h at 37°C, washed 3 times with serum-free DMEM, and transferred into wells of 96-well plates (seeding density 50-100 thousands cells/cm^2), where IMC were then cultured. The medium was changed every 2-3 days, feeder layers were used for 10 days after preparation.

ICM cells from mouse blastocysts were cultured in DMEM with high content of glucose and supplemented with 20% FCS ("HyClone"), β-mercaptoethanol (5×10^{-5} M), 1% essential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10 ng/ml LIF on mitotically-inactivated human fibroblast feeder. After 4 days in culture, potential colonies of growing cells were marked with a fine marker and observed for the subsequent 3 days. To improve the selection of colonies containing stem cells, the cells were subcultured not earlier than 7 days after embryonic IMC disaggregation. Only colonies retaining stable phenotype and homogeneity of the cell population were transferred to the new feeder layer (Fig. 3). The colonies containing heterogeneous cell population (flattened cells at the periphery) were not subcultured. The selected colonies were subcultured with a 3-7-day interval, the number of passages for each culture was recorded. Feeder efficiency was evaluated by the ratio of selected to rejected colonies (in %).

After 3-5 passages the cells were dissociated with collagenase IV (200 U/ml at 37°C), centrifuged for 2 min at 1500 rpm and used for immobilization in the collagen-chitosan matrix.

Type I collagen was extracted from rat caudal tendons with 0.5 M acetic acid. After adjusting the extract to pH 7.0 with ammonium water solution, collagen fibers were precipitated with ethanol (20% v/v). After 30-min centrifugation at 5000*g* and 5°C the collagen sediment was dissolved in 0.1 M acetic acid. Protein content was measured by dry weight after vacuum desiccation. Collagen stock solution with a concentration of 6 mg/ml was used in the experiment. Collagen solution was mixed with culture medium and neutralized with 1 M NaOH.

Chitosan from crab shell (Sigma, 85% deacetylation) was used. The collagen-chitosan hydrogels were prepared as described elsewhere [1]. In 0.1 M HCl chitosan (2% solution) was mixed with culture medium, pH was brought to neutral, 30% Na-glycerophosphate was slowly added. The solution was left at 4°C for 15 min and then mixed with neutralized collagen solution; the mixture was heated to 37°C.

For cell immobilization in the gel, the cell suspension was mixed with the gel-forming solution before heating to 37°C. The proportion between the components was calculated based on experimental conditions: collagen concentration was 0.001-1000 mg/ml, chitosan content 1-20 mg/ml, cell concentration 10^3-10^4 per 1 cm^3 gel.

RESULTS

According to the technique of intact blastocyst transfer into culture medium and their culturing to a stage analogous to early postimplantation develop-

ment, the embryos adhere to the plastic and grow, after which they are disaggregated and embryonic cell culture is isolated. It was shown that embryonic cells after dissociation retain undifferentiated phenotype [3] and can be used as the source of ESC. We found that IMC separated from the trophectodermal layer on days 1-2 grew more slowly than cells isolated at later terms. At the initial stages of stem cell culturing, the best results were observed on days 4-6 in culture followed by enzyme disaggregation of IMC. The mechanism of more effective growth of cells after prolonged culturing on the trophoblast layer remains unknown, but we can hypothesize that during growth on the trophoblast surface IMC increase in number and adapt to the culture medium.

No feeder layer is required for the maintenance of mouse stem cell totipotency, but in our experiments the feeder layer was used as a possible way to improve growth activity of IMC under conditions of low seeding density. Subculturing of IMC was performed every 3-4 days. Different methods of cubculturing were used: pipetting, trypsinization, and the combination of mild enzyme treatment with pipetting. Repeated pipetting broke large cell aggregation (100 and more cells) to smaller aggregations (10-20 cells). Enzyme treatment (0.25% trypsin) for 5 min at 37°C yielded single-cell suspension of IMC. Mild enzyme treatment (0.05% trypsin, 0.02% EDTA, 3 min, 37°C) followed by short-term pipetting yielded aggregation consisting of 3-5 cells.

The maintenance of aggregation size at a level of $\geq$10 cells ensures rapid cell growth, but is associated with high degree of spontaneous cell differentiation (up to 80%). Single IMC did not start proliferate for a long time (up to 1 week) even in the presence of the feeder layer and the percent of CFU was extremely low (about 15%). The best results, *i.e.* high proliferation activity of cells and minimum spontaneous differentiation in culture (~40%), were obtained for aggregations consisting of 3-5 cells. Thus, we can conclude that the conditions of IMC isolation and cubculturing are very important at the early terms of IMC culturing.

The existing methods of creating biomaterials on the basis of collagen-chitosan mixtures had serious limitations determined by irreversible collagen denaturation due to peculiarities of electrostatic interaction of these polymers in solutions. We showed that novel method of stabilization of collage-chitosan matrix by adding polyanion bioactive compounds into this system made it possible to obtain neutral chitosan solutions and did not disturb the formation of collagen fibrils. Ultrastructural study revealed cross-striation typical of collagen (Fig. 4).

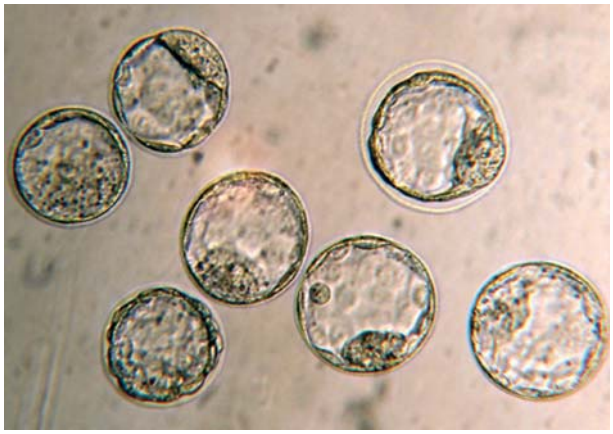

Fig. 1. Mouse embryos at the stage of hatched blastocyst: culturing at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS, ×200.

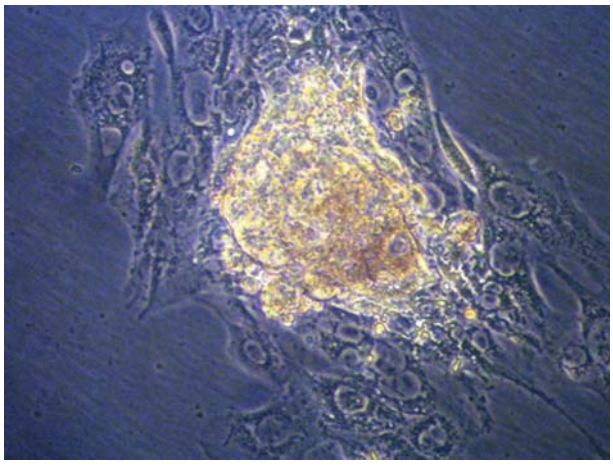

Fig. 2. IMC from blastocysts on the trophectodermal layer: day 5 of culturing in DMEM supplemented with 20% FCS and 10 ng/ml LIF, ×300.

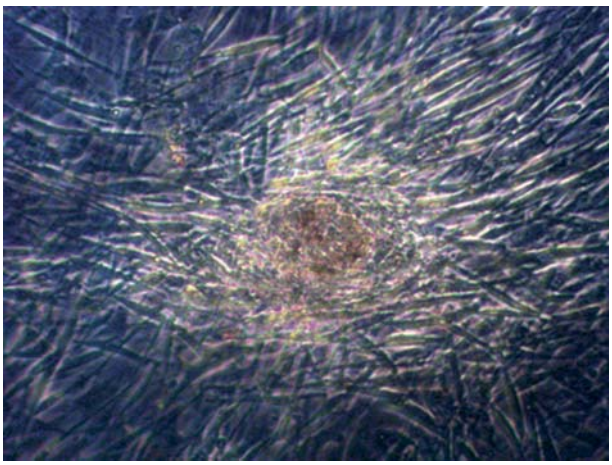

Fig. 3. Culturing of IMC from blastocysts on mitotically-inactivated human fibroblast feeder: day 3 of culturing in DMEM supplemented with 20% FCS and 10 ng/ml LIF, ×200.

The mixture of neutralized collagen and chitosan solutions at low temperatures, physiological pH and ionic strength remains in a dissolved state,

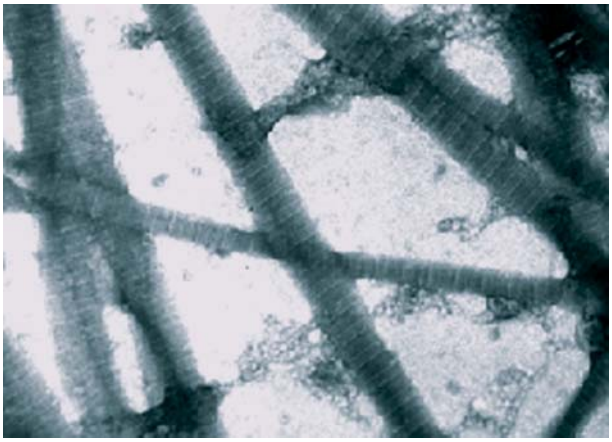

Fig. 4. Ultrastructure of type I collagen gel. Negative contrasting with 1% uranyl acetate, ×60,000.

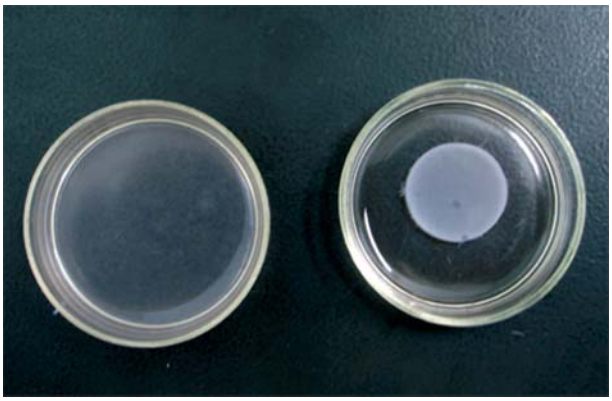

Fig. 5. Contraction of collagen gel (right dish) and the absence of contraction of collagen-chitosan gel (left dish) containing immobilized human fibroblasts.

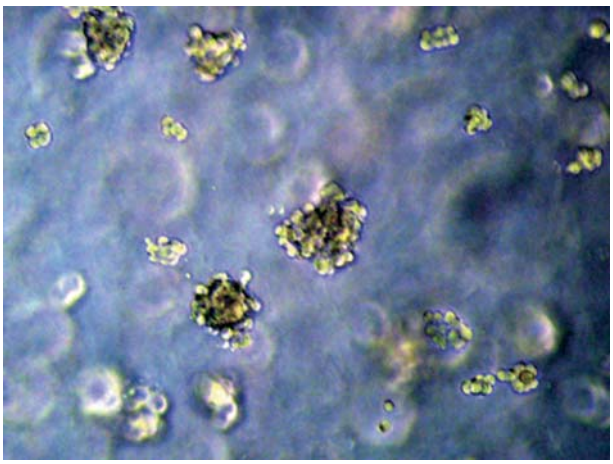

Fig. 6. Mouse ESC immobilized in collagen-chitosan gel matrix (10 mg/ml chitosan, 0.05 mg/ml collagen). Day 7 of culturing in DMEM supplemented with 20% FCS, 5×10^{-5} M β-mercaptoethanol, 1% essential amino acids, and 10 ng/ml LIF, ×300.

which provides conditions for introducing cell suspension and bioactive compounds into this system. After heating to body temperature (at the site of injection) these mixtures form a 3D hydrogels due to hydrophilic-hydrophobic interactions (without potentially cytotoxic cross-linking reagents).

In contrast to collagen gel contracting by several times over the experiment (tens hours), the collagen-chitosan matrix retains its shape throughout the experiment even at a relatively high collagen content (1 mg/ml collagen, 10 mg/ml chitosan, Fig. 5). Highly hydrated gel does not support flattening of fibroblasts and these cells lose their mechanical activity, which probably determined the absence of contraction of collagen-chitosan gels.

Introduction of ESC into collagen-chitosan matrix was also characterized by the absence of cell flattening. However, this is a positive factor for stem cells, because the absence of morphological changes attests to the maintenance of their specific activity. After 3-week culturing in collagen-chitosan gel matrix ESC were viable, had typical shape, and retained their clonogenic activity after seeding to the feeder layer. ESC aggregations consisting of 3-5 cells immobilized in collagen-chitosan gel matrix demonstrated maximum clonogenic activity (about 50%) against the background of far lower proliferation rate compared to culturing on a feeder (Fig. 6). After isolation of stem cells from IMC of solitary blastocysts containing about 100 cells and further culturing of ESC population, the neighbours create necessary conditions for the growth of cell clones.

Our experiments showed that the method of formation of collagen-chitosan hydrogels made it possible to create a cell immobilization system combining characteristics of collagen and chitosan matrices. Stem cell of IMC cultured in collagen-chitosan gel matrix retain their viability and clonogenic activity for a long time, but for evaluation of the possibility of using this matrix for targeted delivery of stem cells the oncogenic and differentiation potential of stem cells cultured under these conditions should be thoroughly studied.

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