Injection Form of Tissue-Engineering Construct on the Basis of Autogenous Chondroblasts for Regeneration of the Cartilaginous Tissue

I. V. Arutjunjan[1,3], A. V. Volkov[1,4], T. B. Bukharova[1,3], P. V. Zelenkov[2], N. A. Konovalov[2], A. A. Rzhaninova[1,3], I. N. Shevelev[2], D. V. Goldshtein[1,3]

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We present an injection form of tissue-engineering construct on the basis of autogenous human chondroblasts for regeneration of the cartilaginous tissue. A material meeting the requirements for the creation of the injection form of an ITC construct (injectable chondrocyte transplantation) was used as the matrix carrier. The developed construct on the basis of autogenous human chondroblasts and homogenized hemostatic gelatin sponge is now at the stage of complex experimental testing.

Key Words: *regeneration of the cartilaginous tissue; human chondroblasts; injection tissue-engineering construct*

Studies in the field of regeneration of the cartilaginous tissue are prompted by high prevalence of intervertebral disk, articular cartilage, and nasal septum pathologies. Cell technologies can be an alternative to low efficient methods aimed at stimulation of regeneration or prevention of degeneration of the cartilaginous tissue. Modern tendencies in the transplantation of viable equivalents of the cartilaginous tissue primarily concern the development of injection forms of tissue-engineering ICT (injectable chondrocyte transplantation) constructs [2,6,12].

For the delivery and fixation of condroblasts in a certain place, biodegradable materials (natural, synthetic, or composite) are used in tissue-engineering constructs as carrier matrices. The scientists creating and applying the carrier matrix for ITC constructs are guided by the following principles: bioinertness of the carrier, its capacity to pass through the injection needle, and sufficient viscosity (for prevention of transplant leakage) [3,13].

Natural carrier matrices can be prepared from blood components (fibrin and platelet-rich plasma, PRP) [14], chitin of invertebrates (chitosan), and plant raw material (alginate) [9]. Some investigators create constructs including extracellular matrix proteins, e.g. ostein (demineralized bone matrix) [1], elastin [7], and gelatinized type 1 collagen [10].

Among synthetic materials, polylactide microspheres are most often used [4]. Polyethylene glycol and hyaluronic acid polyesters are used in the form of gels [8].

The present work is devoted to the development of a tissue-engineering construct on the basis of autogenous human chondroblasts and a carrier matrix consisting of a material registered by Federal Service on Surveillance in Healthcare and Social Development of Russian Federation and meeting

[1]ReMeTeks Company; [2]N. N. Burdenko Institute of Neurosurgery; Russian Academy of Medical Sciences; [3]Medicogenetic Research Center, Russian Academy of Medical Sciences; [4]Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** remetex@yandex.ru. I. V. Arutjunjan.

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all requirements for the creation of an injection construct.

MATERIALS AND METHODS

Isolation of primary human chondroblast culture. For isolation of chondroblasts we used biopsy material obtained during surgery for excision of sequestrated intervertebral disk hernia. Cultures obtained from 9 patients (3 women and 6 men, mean age 37 years) operated for excision of intervertebral disk hernia at N. N. Burdenko Institute of Neurosurgery and tested for the presence of viral and bacterial infections were used.

The material (a fragment of annulus fibrosus of intervertebral disk endplate) was transported to the laboratory within 1 h after surgery in a special transporting container. The cartilaginous tissue was maximally cleansed from the connective tissue, minced with scissors, and incubated for 1.5-3 h in enzyme solution containing 0.02% pronase E (Sigma), 0.1% collagenase II (PanEko), and 0.1 hyaluronidase (Sigma) in 1:1:1 ratio. After incubation, the total number of isolated cells and their viability were evaluated by exclusion of 0.4% trypan blue.

The material was then transferred into Nunc flasks with complete nutrient medium DMEM/F12 (1:1; PanEko) supplemented with FCS (up to 20%, HyClone-Perbio), L-glutamine (4 mM, PanEko), amikacin (500 mg/liter, Sintex AKO), insulin-transferrin-selenite (to 2%, PanEko); $1\text{-}2\times10^5$ cells per 1 ml medium.

Immunocytochemical studies. Synthesis of extracellular matrix by chondroblasts was evaluated by cytochemical staining with mouse monoclonal antibodies to type 1 and type 2 collagen and aggrecan (Abcam). Proliferative capacity of chondroblasts was evaluated by the method recommended by the manufacturer using rabbit polyclonal antibodies to Ki67 (Abcam).

Evaluation of cytotoxicity. Cytotoxicity of the gelatin sponge was assessed using MTT test. The test is based on the capacity of mitochondrial succinate dehydrogenase to reduce light yellow MTT-tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) to insoluble dark-colored formazan. Formazan crystals were extracted from cells with organic solvents (DMSO).

The cells were seeded to 96-well plates at a density of 10,000 cells per well. After cell adhesion and flattening, a gelatin sponge fragments (~1 mm^3) were placed into wells; control wells contained no sponge. After 1, 2, or 7 days, 0.5 mg/ml MTT (PanEko) was added and the plates were incubated for 2 h at 37°C under visual control. The reaction was stopped by removing the medium containing MTT. Formazan was eluted with DMSO (PanEko) for 20 min (on a shaker at 200 rpm).

Adsorption of formazan was evaluated by measuring optical density of the eluate at λ=540 nm and standardizing it to the background value measured at λ=620 nm. Fluoroscan Ascent plate reader (Labsystems) was used.

RESULTS

Isolation and characteristics of human chondroblast culture. The isolated cells adhere to plastic within 24-48 h. Some cells retained spherical shape, while others started to flatten on the substrate (Fig. 1, *a*).

After 1.5-2 weeks, the culture attained confluence and the cells were subcultured not more than 3-4 times. The total number of passages did not exceed 3. All 9 autogenous chondroblast cultures were presented by small uniform polygonal cells (<10-12 μ) with few processes, centrally located nucleus, and weakly granular cytoplasm (Fig. 1, *b*).

In various cultures, the percent of cells synthesizing type 1 collagen varied from 21 to 28% and the percent of cells producing type 2 collagen and aggrecan varied from 82 to 92% (Fig. 2, *a*). Under conditions of long-term culturing with high cell density, actively proliferating chondroblasts spontaneously formed aggregates characterized by enhanced synthesis of extracellular matrix (aggrecan and type 2 collagen, Fig. 2, *b*).

Evaluation of proliferative activity of chondroblasts showed that about 40% cells were in a state of active proliferation (*i.e.* in G_1, S, G_2, or M phases of the cell cycle) immediately after seeding (Fig. 3), while other 60% cells did not express Ki67, *i.e.* were in a resting state. The percent of resting cells attained 100% during long-term culturing at high cell density.

Carrier matrix. When choosing the matrix for the tissue-engineering construct meeting the purposes of our study, we focused on two requirements to the carrier matrix: cell integrity during low-invasive (injection) route of administration and cell fixation at the site of application. Analysis revealed considerable drawbacks of the majority of widely used matrixes for injection application such as toxicity, impossibility of biodegradation, instability, *etc*.

In our laboratory, we previously used a hemostatic gelatin sponge as the basis for the construct. Here we used Spongostan hemostatic gelatin sponge consisting of 1-cm^3 sterile sponge blocks. The choice of this sponge as the carrier was based on the analysis of its propereties: the hemostatic gela-

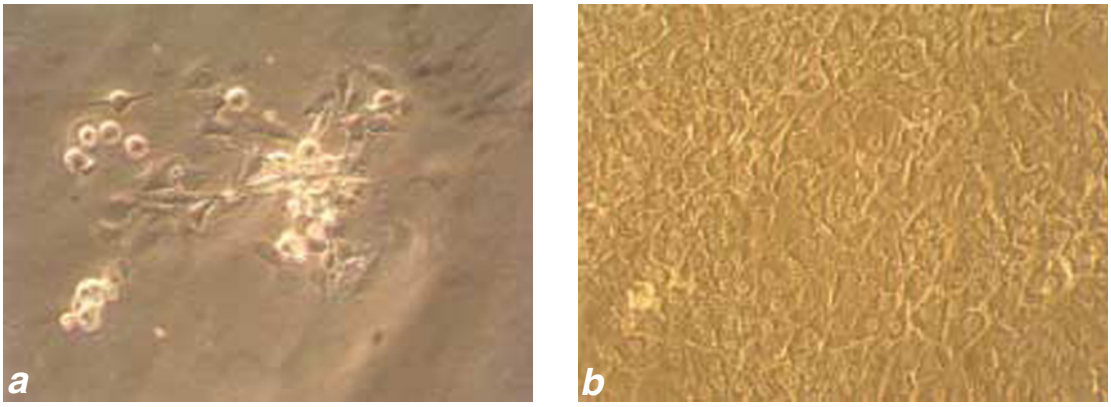

Fig. 1. Human chondroblast culture. *a*) cell adhering to the substrate, ×400; *b*) confluent monolayer, ×200.

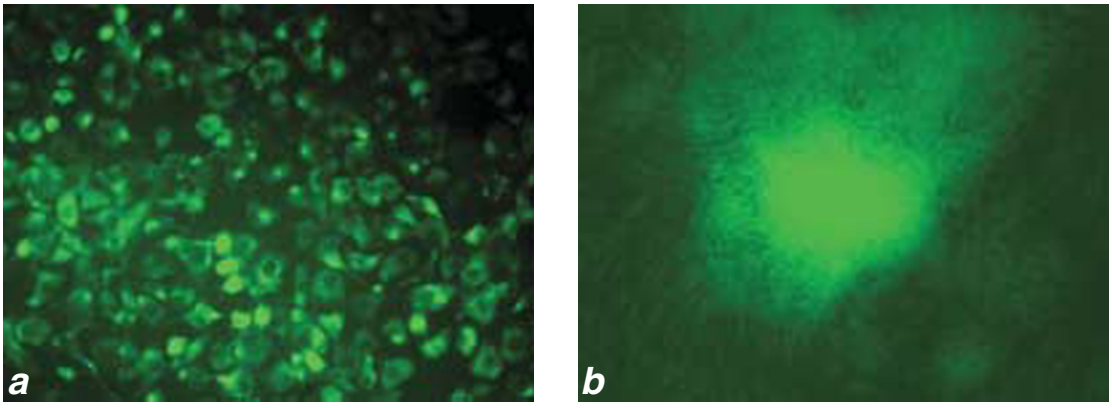

Fig. 2. Synthesis of aggrecan in chondroblast culture. *a*) synthesis of aggrecan (immunofluorescence, ×200); *b*) enhanced synthesis of extracellular matrix (aggrecan) in chondroblast aggregates (immunofluorescence, ×100).

tin sponge is listed in the Drug Register of the Russian Federation, it is not cytotoxic, exhibits high adhesion to cells and tissues, is biologically inert, and contains no additional components (antibiotics *etc*.). This sponge is stable, elastic, sterile, and water-insoluble, it is resorbed in tissues within 3-5 weeks and exhibits affinity to body tissues.

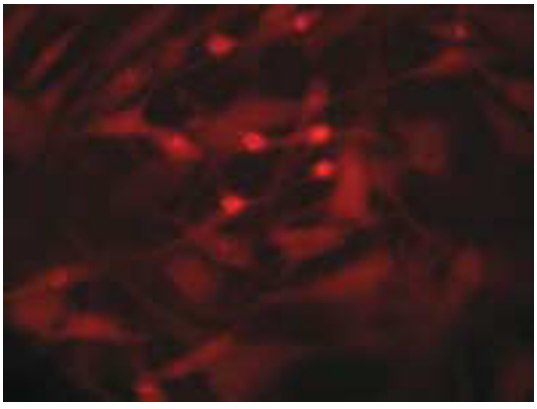

Fig. 3. Expression of Ki67 by chondroblast at the stage of subconfluent monolayer (immunofluorescence, ×400).

The sponge exhibited no toxic properties within 1 week and even slightly stimulated chondroblast proliferation. Two-week culturing showed that parameters of control and experimental wells became similar after 2 days; on day 7, the percent of viable cells decreased compared to the control, but this decrease did not exceed 5% (Fig. 4).

For preparing the injection form, the sponge blocks were minced with a dispersion homogenizer. We used different regimens of homogenization for preparing gelatin sponge particles of different sizes. These particles represented a 3D interlacing of planes and thin lamellar structures (Fig. 5, *a*). Further studies showed that the optimal size of particles varied from 100 to 500 μ, these particles were obtained at 5000-6000 rpm homogenizer knife rotation rate.

Preparation and characteristics of tissue-engineering construct. After obtaining a required amount of biomaterial (20-30 mln. chondroblasts), the cells were harvested with trypsin-versene and precipitated by centrifugation at 1100 rpm for 10 min. Gelatin sponge blocks were minced with a dispersion homogenizer (5000-6000 rpm). The car-

rier and suspended cell pellet were mixed and placed in a CO_2 incubator under standard conditions. After 1-2-day incubation, the tissue-engineering construct was precipitated by centrifugation at 500 rpm for 10 min.

A total of 50-200 cells adhered to a standard structural unit of the carrier 3 h after mixing the components of the tissue-engineering construct; cell viability was determined by trypan blue (0.4%) exclusion (Fig. 5, *b*).

At this stage, some adherent chondroblasts started to flatten on the substrate (Fig. 6, *a*), after 1 day, these cells constitute 80%, while after 2 days the construct represented a carrier coated with flattened cells (Fig. 6, *b*). Thus, the data of MTT test and kinetics of cell flattening suggest that the 2-day period is optimal for the creation of the construct.

Stability of the tissue-engineering construct. For evaluation of the stability of the obtained construct to external factors corresponding to injection conditions, it was transferred into a syringe, allowed to stay at 4°C for 3 h, and passed through a 0.6-mm needle. Cell viability evaluated by trypan blue exclusion practically did not change and the cells retained flattened shape. Thus, the obtained tissue-engineering construct was resistant to exter-

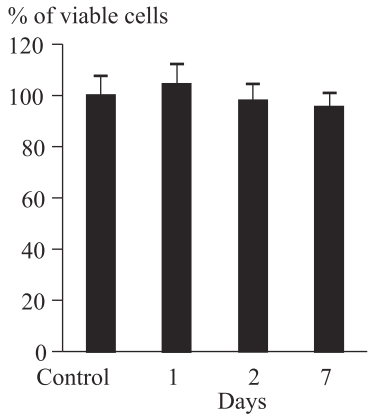

Fig. 4. Evaluation of cytotoxicity of gelatin sponge by MTT test.

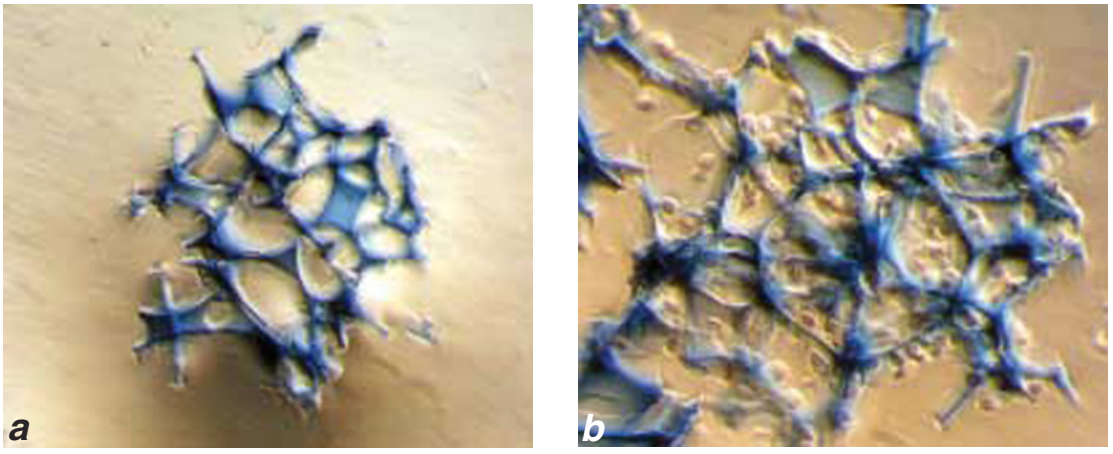

Fig. 5. Standard structural unit of the carrier. *a*) appearance of a structural unit of the carrier, *b*) evaluation of viability of cells immobilized on the carrier. Trypan blue staining (0.4%; ×100).

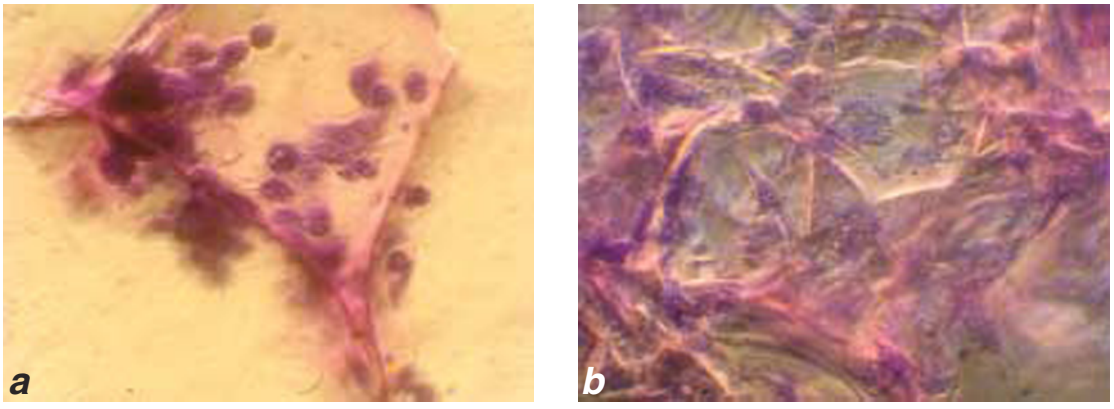

Fig. 6. Dynamics of chondroblast flattening on the carrier surface after mixing the construct components. *a*) after 3 h; *b*) after 2 days. May-Grunewald staining, ×400.

nal physical factors, which attests to the possibility of using the injection form of the preparation.

It should be noted that the results of injection of a suspension of nonadherent cells and transplantation of a tissue-engineering construct were principally different. Experiments on animals showed that cell death after injection of cell suspension can attain 90% due to mechanical injury and the absence of proper microenvironment [11]. Poor survival and uncontrolled diffuse migration of cells into the adjacent tissue considerably decreased the efficiency of this transplantation [5]. After injection of the tissue-engineering construct, the cells adhering to the carrier are fixed at the site of injection, while elastic carrier protects them from mechanical injury. Moreover, 3D tissue-engineering construct is in fact a histion, which excludes sharp changes in microenvironment impairing functional activity of the transplanted cells.

Thus, we create a tissue-engineering construct for regeneration of the cartilage tissue fully meeting the required parameters and prepared an injection form of the preparation, which makes possible to use low invasive methods of transplantation. The use of this tissue-engineering construct for medical purposes opens wide prospects.

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REFERENCES

1. R. Q. Brown, A. Mount, and K. J. Burg, *J. Biomed. Mater. Res. A.*, **74**, No. 1, 32-39 (2005).
2. C. De Bie, *Regen. Med.,* **2**, No. 1, 95-97 (2007).
3. J. Elisseeff, // *Expert Opin. Biol. Ther.*, **4**, No. 12, 1849-1859 (2004).
4. Y. Hong , Y. Gong, C. Gao, and J. Shen, *J. Biomed. Mater. Res. A.*, **85**, No. 3, 628-637 (2008).
5. I. Kutschka, I. Y. Chen, T. Kofidis, *et al.,* Circulation. **114**, Suppl. 1, 174-180 (2006).
6. M. Manfredini, F. Zerbinati, A. Gildone, and R. Faccini, *Acta Neurol. Belg*., **73**, No. 2, 207-218 (2007).
7. M. K. McHale, L. A. Setton, and A. Chilkoti, *Tissue Eng*., **11**, No. 11-12, 1768-1779 (2005).
8. P. A. Revell, E. Damien, L. Di Silvio, *et al*., *J. Mater. Sci. Mater. Med.*, **18**, No. 2, 303-308 (2007).
9. J. C. Schagemann, E. H. Mrosek, R. Landers, *et al., Cells Tissues Organs.*, **182**, No. 2, 89-97 (2006).
10. A. H. Shikani, D. J. Fink, A. Sohrabi, *et al., Am. J. Rhinol*., **18**, No. 2, 105-112 (2004).
11. C. E. Sortwell, M. R. Pitzer, T. J. Collier, *Exp. Neurol.*, **165**, No. 2, 268-277 (2000).
12. J. J. Wood, M. A. Malek, F. J. Frassica, *et al., J. Bone Joint Surg. Am*., **88**, No. 3, 503-507 (2006).
13. T. B. Woodfield, J. M, Bezemer, J. S. Pieper, *et al., // Crit. Rev. Eukaryot. Gene Expr.*, **12**, No. 3, 209-236 (2002).
14. J. W. Xu, V. Zaporojan, G. M. Peretti, *et al., J. Plast. Reconstr. Surg*., **113**, No. 5, 1361-1371 (2004).