Study of the Safety of Transplantation of Cultured Autologous Human Chondroblasts

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Study of tumorigenic activity of cultured human chondroblasts intended for autologous transplantations showed that subcutaneous injection of human chondroblast suspension in a dose of 10^7 cells/0.2 ml did not cause the development of tumors in C.B-17 SCID immunodeficient mice over 12 weeks. Subcutaneous formations of compact cartilaginous consistency persisted at the site of chondroblast transplantation in all animals; these formations gradually shrank. Subcutaneous injection of tumor cell suspension (A204 human embryonic sarcoma) to C.B-17 SCID mice under the same conditions caused 100% development of tumors (poorly differentiated sarcomas) by day 14.

Key Words: *tumorigenic activity; human autologous chondroblasts; transplantation*

Introduction of cell technologies into clinical practice necessitates solving some problems of safety of therapeutic use of cell material, primarily the risk of tumor development because of probable spontaneous malignant transformation of cells in culture. This risk seems to be substantial, if we review the data on spontaneous tumor transformation of different human and animal cell strains, including mesodermal cells [1,9]. Cell expansion in culture is in the majority of cases an obligatory step in preparation of the material for transplantation. In addition to cell aging as the main cause of malignant transformation, we cannot rule out the negative impact of some *ex vivo* manipulations, for example, genetic modification [6].

Therefore, any somatic cells subjected to culturing and the above manipulations and intended for clinical use should be tested for tumorigenic activity (capacity to form a tumor after transplantation into an immunologically tolerant organism). According to modern requirements, these studies are obligatory for all cell strains used for the production of biological preparations.

Degenerative diseases of the spine constitute 60-65% of all spinal diseases. Surgical methods widely used for the treatment of these diseases are not pathogenetic and do not arrest the degenerative process in the intervertebral disks. Cell technologies based on transplantation of autochondroblasts can become an alternative to ineffective methods aimed at stimulation of cartilage tissue regeneration [7,8]. However, we failed to find published data on the safety of using cultured chondroblasts in practical medicine.

Tumorigenic activity of cell strains is traditionally studied on nude (Nu/Nu) mice lacking T cells. We selected the SCID model for our study. These animals are characterized by severe combined immunodeficiency without production of not only T, but also B cells. Combined immunodeficiency of SCID mice is caused by a genetic deficiency of some elements of Ig and T-cell receptor genes, which results in disor-

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ders in differentiation of T and B progenitor cells. In addition, SCID mice cannot produce antibodies to common antigens and reject allogenic and xenogenic transplants.

Except these immunological characteristics, SCID mice are normal: they have normal composition and functions of blood cells other than lymphocytes, including natural killers (NK), macrophages, and granulocytes. They have lymph nodes and the thymus, though of lesser size. In addition, SCID mice are an inbred strain, which is an advantage in comparison with nude mice, usually heterozygous because of common breeding.

We carried out a preclinical study of tumorigenic safety of cultured chondroblasts on a C.B-17 SCID laboratory model.

MATERIALS AND METHODS

Human chondroprogenitor cells were isolated from a fragment of annulus fibrosus of intervertebral disk end-plate. The cultures were obtained from patients (n=5) subjected to planned resection of the intervertebral disk hernia at N. N. Burdenko Institute of Neurosurgery. All specimens were tested for viral and bacterial infections.

The material (annulus fibrosus of intervertebral disk end-plate) was delivered to the laboratory in a special container within 1 h after surgery. The cartilage tissue was maximally thoroughly cleansed from the connective tissue, fragmented with scissors, and incubated with enzymes: 0.02% pronase E (Sigma), 0.1% collagenase II (PanEco), and 0.1% hyaluronidase (Sigma) in 1:1:1 proportion for 1.5-3 h. After incubation, the total count of isolated cells was evaluated and their viability was tested by staining with 0.4% trypan blue.

The cells were cultured in DMEM/F12 growth medium (1:1, PanEco) with 20% FCS (HyClone-Perbio), 2 mM L-glutamine (PanEco), 0.5% mg/ml amikacine (AKO Synthesis), 2% insulin-transferrin selenite (PanEco). Inoculation density was 100,000-200,000 cell/ml. After formation of a confluent monolayer, the culture was reinoculated 1:3; the total number of passages did not exceed 5, the number of cell doubling *in vitro* did not exceed 15. The resultant cultures were cryopreserved in the protective medium, consisting from autologous serum with 10% DMSO, and stored in liquid nitrogen. Each culture was stored in a separate container. Two hours before injection of chondroblast culture and control tumor strain, the cultures were defrosted, the cells were counted and resuspended in Hanks' solution to a concentration of 5×10^7 cell/ml.

Human embryonic A204 sarcoma (ATCC® No. HTB-82™) served as the positive control. The culture grew in DMEM with 10% FCS. The medium was replaced twice a week; the culture was reinoculated 1:10.

According to published data, A204 strain is tumorigenic in nude or newborn mice immunosuppressed with antithymocytic serum; injected subcutaneously, these cells form small malignant tumors [2,3].

Tumorigenic activity of the cultures was tested on C.B-17 SCID male mice (Charles River Laboratories). At the beginning of the study, the animals were aged 6 weeks with the mean body weight of 19.9 g. The animals were divided into 6 groups, 10 per group: 5 experimental (for each of the tested cultures) and 1 control. Experimental animals were injected with chondroblast suspension (10^7 cells/0.2 ml, single subcutaneous injection in the right lateral part of the trunk). Positive controls were injected with suspension of A204 human embryonic sarcoma cells in the same dose and volume.

The animals were daily weighed during the first 2 weeks of the experiment and then every other day. The sites of chondroblast suspension injection and inoculation of A204 tumor cells were regularly examined and palpated. All bulky formations (length×width×height) were measured with a gage and ruler every 3 days (frequency needed for the formation of nodules) until sacrifice. Euthanasia was carried out in a CO_2 chamber.

Controls (n=10) with tumor nodules were sacrificed on day 14 after injection of tumor cells. Experimental animals were observed for a longer period: 5 animals for 21 days, the rest for 12 weeks after injection of the studied cells. After sacrifice, autopsy was carried out with visual and histological evaluation of tissues at the site of cell injection and of possible metastases. The following material was collected for histological study: tumor nodules from control animals; skin sites with subcutaneous fat at the site of cell suspension injection; fragments of the brain, lung, liver, spleen, kidney, inguinal and axillary lymph nodes on the side of injection, and all macroscopically changed tissues from experimental animals. The material was fixed in 12% neutral formalin, subjected to histological processing, and embedded in paraffin; histological sections were stained with hematoxylin and eosin.

The presence of tumors, their number, size, and time of development, the presence of regional and distant metastases, atypical cells, signs of invasive growth, and mitotic activity of cells served as the tumorigenicity criteria for chondroblasts and A204 human embryonic sarcoma cells.

The study was carried out in accordance with the GLP (Good Laboratory Practice) international standards, regulating the norms, rules, and instructions aimed at obtaining coordinated reliable results of laboratory studies. Development of methods of experimental studies of the tumorigenic potential of cell trans-

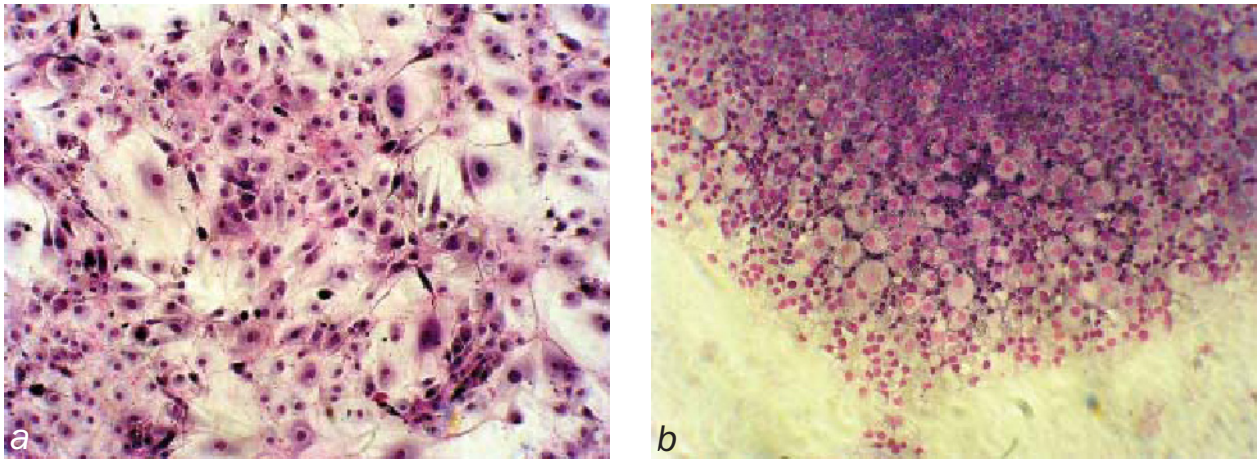

Fig. 1. Morphology of the studied cultures (Popenheim staining, ×200). *a*) human chondroblast culture (experimental group A); *b*) A204 cells.

plants was based on the recommendations of Food and Drug Administration (USA) for preclinical studies of cell strains used for the production of biological preparations and for evaluation of tumorigenic activity of tumor cell substrates [4,5].

RESULTS

The studied cultures were homogenous and consisted of small polygonal cells with short processes, central nuclei, and slightly granular cytoplasm (Fig. 1, *a*). During high-density culturing, the cells spontaneously formed groups with synthesis of extracellular matrix proteins (aggrecan and collagen-2) characteristic of cartilage tissue. Chondroblast strains from 5 adult donors cryopreserved after passage 5 were studied. The cultures originating from the donors were denoted as A, B, C, D, E.

The positive control A204 strain was a colony of epithelium-like cells. Some large round cells were in a half-adhesive state (Fig. 1, *b*).

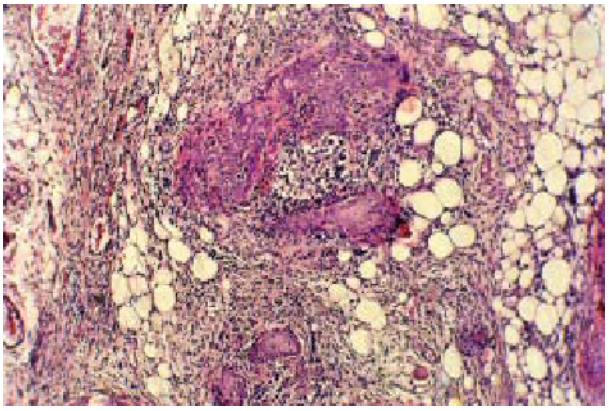

Fig. 2. Invasive growth of A204 sarcoma in subcutaneous fat. Elements of bone and cartilage differentiation. Sections stained with hematoxylin and eosin (×100).

All control animals developed subcutaneous nodules at the site of injection on day 3 after injection of A204 human embryonic sarcoma cell suspension; the mean size of these nodules was 45.6±2.7 mm^3. Later these formations slowly increased in size in some animals, while in others they remained virtually unchanged. On days 7 and 11, two animals were sacrificed because of grave status and body weight loss. By the moment of euthanasia, subcutaneous nodules were palpated in them at the site of cell suspension injection (20 and 48 mm^3, respectively).

The status of other animals was satisfactory; body weight somewhat increased. By day 14, small subcutaneous tumors were palpated in all control animals; the mean volume of these tumors was 39.3±12.5 mm^3 by the moment of euthanasia.

Macroscopic examination revealed small subcutaneous tumors in all mice. The tumors were soft, elastic pink-white, mainly solitary nodules, easily detached from the skin. In one case, the tumor consisted of 2 nodules and in two cases it tightly coalesced with the skin.

Histological study showed solid tumors in all 10 animals. By morphological picture, the tumors corresponded to embryonic (mesenchymal) sarcoma with atypical chondroid elements (Fig. 2). Large cartilage structures (of the hyaline cartilage type) with oxyphilic main substance containing solitary round cartilage cells in its lacunae, were detected in amorphous oxyphilic main substance and among numerous fibroblast-like cells with cellular and nuclear polymorphism, sometimes forming the syncytium sites. In some places, the tumor tissue contained many capillaries and sites of the adipose tissue. Invasion of tumor cells into subcutaneous fat, derma, and between the subcutaneous muscular bundles was seen; mitotic figures were often detected. No transversely striated cells were seen among the tumor cells.

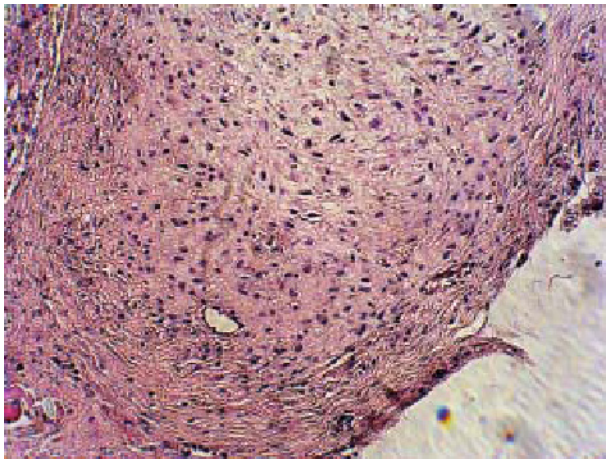

Fig. 3. Histological picture of the site of chondroblast culture injection: 3 weeks after transplantation. Sections stained with hematoxylin and eosin (×100).

Macroscopic examination of autopsy material on day 14 after inoculation of A204 sarcoma cells showed no apparent metastases in the regional lymph nodes, brain, heart, lungs, liver, kidneys, and spleen of all 10 mice. Autopsy of 2 animals sacrificed on days 7 and 11 showed signs of enteritis in the abdominal cavity (swelling and greenish coloring of the intestinal loops). No other apparent signs were detected in the viscera. Hypoplasia of the spleen and lymph nodes, a characteristic feature of SCID mice, were seen in all animals.

Small flat yellowish subcutaneous formations of compact consistency differing by the volume (from 2.50±0.32 mm^3 in group D to 24.70±6.38 mm^3 in group A) were macroscopically detected soon after chondroblast transplantation at the site of injection in animals of all experimental groups (Table 1).

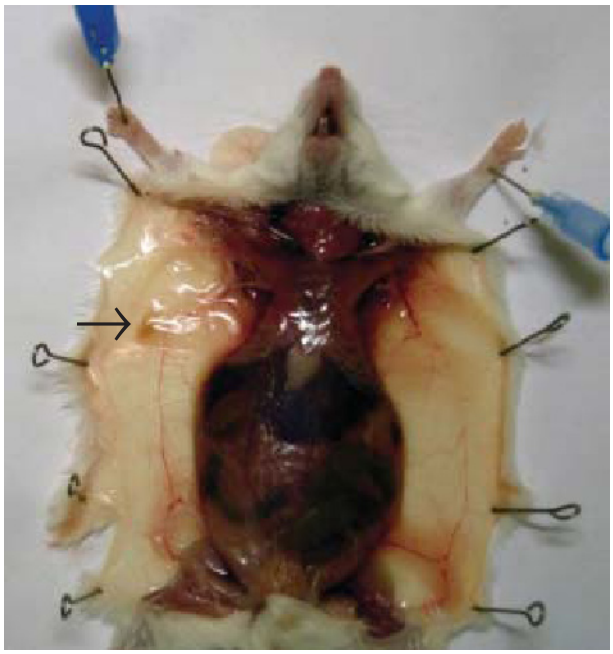

Fig. 4. Subcutaneous formation of compact cartilage-like consistency at the site of chondroblast transplantation (shown by an arrow) in a mouse from group A: 12 weeks after transplantation.

Histological study of the site of chondroblast injection on day 21 revealed small mainly round formations (transplants) in the subcutaneous adipose tissue between the derma and subcutaneous muscles, sometimes even between muscles, in the majority of mice. Many chaotic connective tissue cells (round, polygonal, spindle, and stellate) were seen in the mesenchyma-like tissue in amorphous oxyphilic slightly fibrous interstitium. A small part of cells had signs of degeneration. Round cells with "shrunk" cytoplasm, resembling chondrocytes, were seen in some places in the interstitial lacunae. A well-formed capsule from several layers of collagen fibers and fibroblasts between them was a characteristic feature; capillaries were seen outside the capsule and in some cases in it. No mitotic figures or cell invasion in the adjacent tissues were detected (Fig. 3). A site of young adipose tissue was seen at the site of transplantation in one mouse.

Macroscopic examination 12 weeks after transplantation of chondroblasts showed smaller (in comparison with early period of observation) subcutaneous formations in all mice. These formations were whitish or yellowish, usually of compact cartilage-like consistency (Fig. 4).

Histological study of the injection sites during this period showed that the transplants were located in the subcutaneous fat between the derma and myofibrils, were mainly round, consisted of oxyphilic slightly fibrous substance with incorporated cells, the number of cells being lower than during previous periods. Round, spindle, polygonal cell were embedded in the thickness of the interstitium. In some places round cells with shrunk cytoplasm were located in the interstitial lacunae and resembled chondrocytes. The greater part of cells exhibited signs of degeneration. Macrophages with detritus particles were detected in the cytoplasm. A wide connective tissue capsule consisting of coarse collagen fibrils with few fibroblasts and fibrocytes between them was seen at the periphery of formations in all cases. In some animals, the structures resembling a hyaline cartilage, in some places with bone cords and with foci of myeloid hemopoiesis between these cords, were seen at the site of injection of chondroblast suspension between subcutaneous muscles (Fig. 5).

The transplants completely resolved and were not detected macroscopically in 30% mice in groups C, D, and E.

Autopsy of 25 animals of experimental groups on day 21 revealed no apparent metastases in the regional axillary and inguinal lymph nodes, spleen, brain, heart, lungs, liver, kidneys, and abdominal cavity. Plethoric

pulmonary tissue was seen in 4 animals, hypoplastic spleens and lymph nodes (a characteristic sign of SCID mice) were seen in all animals.

Histological studies of the regional lymph nodes, lungs, liver, spleen, kidneys, and brain revealed no metastases. Nonspecific changes (uneven venous plethora, inflammatory peribronchial infiltration) were detected in the lungs of 5 animals, and a site of atelectasis in 1 mouse. Poorly expressed lymphoid follicles, blurred interface between the cortical and medullary layers were noted in the regional axillary and inguinal lymph nodes. Evenly distributed lymphoid elements in the white pulp, no lymphoid follicles, and plethoric red pulp in some places were seen in the spleen in all animals. Pyelonephritis symptoms were detected in the kidneys in 1 mouse, degenerative changes in the tubules in 2.

Autopsy of the remaining 25 animals on day 84 of the experiment showed no apparent metastases in the regional axillary and inguinal lymph nodes, spleen, brain, heart, lungs, liver, and kidneys. Whitish deposition on the pericardium was detected in 2 animals, and plethoric lungs in 3 others. Splenic and lymph node hypoplasia was observed, as previously.

Histological studies of the regional lymph nodes, lungs, liver, spleen, kidneys, and brain showed no metastases. Nonspecific changes in the lungs (venous plethora of the parenchyma, inflammatory peribronchial infiltration, emphysema foci) were seen in 3 animals. Histological examination of the heart tissue in animals with macroscopic changes in the pericardium detected signs of pericarditis (leukocytic infiltration of the pericardium and adjacent myocardial layers; fibrin deposition). Blurred boundary between the cortical and medullary substance and poorly expressed lymphoid follicles were seen in the lymph node tissue. Even distribution of lymphoid elements in the white pulp and poorly expressed lymphoid follicles in the spleen were seen in all animals.

Hence, compact subcutaneous nodules 1-5 mm in diameter were palpated in the majority of animals soon after transplantation at the site of cell suspension injection. Later these subcutaneous nodules at the site of cell suspension injection gradually shrank; in some animals these formations could no longer be palpated. By the end of observation, the largest formations (11.20±6.65 mm^3) were seen in experimental group 1 (chondroblasts A) and the least ones (1.00±0.64 mm^3) in experimental group 4 (chondroblasts D).

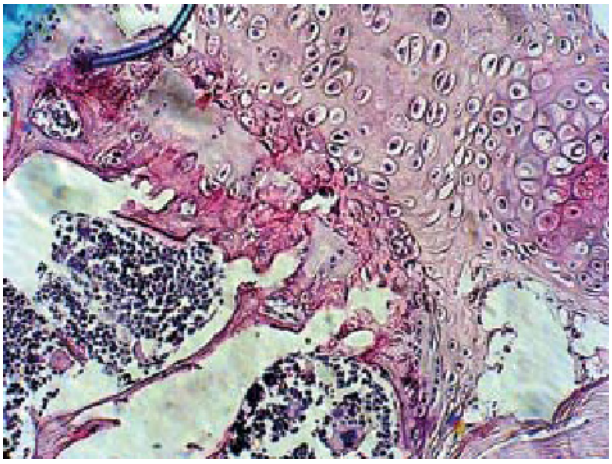

Fig. 5. A hyaline cartilage-like structure in a transplant from group B. Foci of myelin hemopoiesis at the periphery of formation. Hematoxylin and eosin staining (×100).

The results indicate that subcutaneous injection of human chondrocyte suspension (10^7 cells/0.2 ml) causes no tumor development in C.B-17 SCID immunodeficient mice over 12 weeks (84 days). Macroscopic and histological studies of the viscera and brain detected no metastases.

Subcutaneous injection of A204 human embryonic sarcoma cells (10^7 cells/0.2 ml) to immunodeficient mice caused the development of tumors, poorly differentiated sarcomas (100% in control group), by day 14 of observation. Macroscopic and histological study of the viscera and brain showed no metastases.

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TABLE 1. Mean Volume of Tumors and Subcutaneous Nodular Formations in Experimental Groups during Different Periods of Observation ($M\pm m$; mm^3)

Period of observation	Control	Experimental groups				
		series A chondroblasts	series B chondroblasts	series C chondroblasts	series D chondroblasts	series E chondroblasts
14 days	39.3±12.5	24.70±6.38	5.70±1.62	9.10±2.05	2.50±0.32	15.60±3.46
21 days	—	24.20±5.71	8.10±2.53	9.30±3.63	4.75±1.46	18.90±6.05
12 weeks	—	11.20±6.65	4.60±3.22	1.40±0.86	1.00±0.64	2.75±1.97

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