SELECTED LECTURES

ON SPINE SURGERY

NEW PLASTIC MATERIAL FOR THE TREATMENT OF VERTEBRAL PATHOLOGY AND NOT ONLY...

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The paper presents results of the author's multi-year research in the field of cellular technologies. The differentiation stages of the chondro-osseous graft are described based on the findings of morphological, ultrastructural, and molecular genetic studies. The stages of cell transformation, dynamics of synthetic processes and matrix formation are identified. Hypotheses of the histogenesis of initial stages of different types of bone tissue, and the formation of microcirculatory and vascular beds in the process of osteogenesis are suggested. Experimental testing of regenerative potentialities of the bone graft in animals has shown that it is a primitive bone tissue with a high potency to proliferation, formation and differentiation into definitive bone tissue within a transplant zone.
Key Words: bone tissue, chondro-osseous graft, osteogenesis.

Please cite this paper as: *Zaidman AM. New plastic material for the treatment of vertebral pathology and not only... Hir. Pozvonoc. 2018;15(2):91–97. In Russian. DOI: http://dx.doi.org/10.14531/ss2018.2.91-97.*

The knowledge of bone histogenesis is one of the important issues, which provides the opportunity to regulate osteogenesis.
A.Ya. Friedenstein

The fascinating dance of a ballerina or the magical music of Chopin, Shostakovich, Tchaikovsky, Mozart, and Rachmaninov, which cascades from the violins of Oistrakh, Paganini, and Kogan or the Richter piano, is not only a combination of the mind and talent but also the highest level of performing skills that include mastering the subtlest nuances of the musculoskeletal system functioning. The modern musculoskeletal system has traveled a long evolutionary way. Everything began in antiquity with the appearance of scales and surface ossicles in echinoderms as protective structures [8]. As organisms became more complex, the bone system evolved to support, as a part of the endoskeleton, the body. The protective nature has retained even in modern mammals: bony sheaths cover two important organs, the brain and spinal cord. Since then, the functions of bone tissue have significantly diverged. First of all, bone tissue is an essential component for the normal development of cells of the bone marrow that is the source of renewal of the internal environment system in mammals [10]. Reproduction and differentiation of stem hematopoietic cells towards formation of hematopoietic cells and blood elements require close contact with the bone undergoing active osteogenesis [3]. This is also confirmed by the formation of ectopic bone tissue that is invariably populated by hematopoietic cells to give rise to a new bone marrow organ. Involvement of hematopoietic tissue in the histogenesis is an important function of bone tissue.

At present, bone marrow injections are known to save lives of children and adults with genetic pathologies associated with disrupted unity of the bone and hematopoietic systems.

Bone tissue is a depot of mineral metabolism. In a 70 kg man, bone crystals (hydroxyapatite crystals) contain 1,200 g of calcium; the total surface of bone crystals is 400,000 m^2 and provides an extensive territory for ion exchange. Sometimes, this is not sufficient for a normal blood level of calcium (Ca^{++}), and the Ca^{++} depots are mobilized from internal parts of bone crystals.

The skeleton (bone) is not only the depot of mineral salts but also the buffer system involved in maintaining the Ca^{++} ion concentration. One of the functions of bone tissue is continuous maintenance of the bone formation process. This means that the bone throughout the ontogenesis is a continuously renewing system that provides the formation of bone and hematopoietic tissue in the necessary amount.

Of course, bone tissue injury, defects, and surgical interventions affect metabolic processes in the body; therefore, restoration of defects or replacement therapy for bone tissue loss is the most important problem in restorative medicine.

Correction of bone tissue defects, in particular skull defects in warriors, was undertaken as early as in ancient times. The used materials included gold, silver, and even walnut shells. Restorative surgery was pioneered by N.I. Pirogov who first in the world performed osteoplastic surgery for severe foot damage in 1852. He sutured a piece of the calcaneus to a cut of the lower leg bones. Heterotransplantation was pioneered by Ambroise Pare in the 16th century, who replaced a bad tooth of a princess by a good tooth of her maid [7]. There were reports of successful experiments of Prof. A.G. Lapchinskiy who grafted limbs and even a head in dogs.

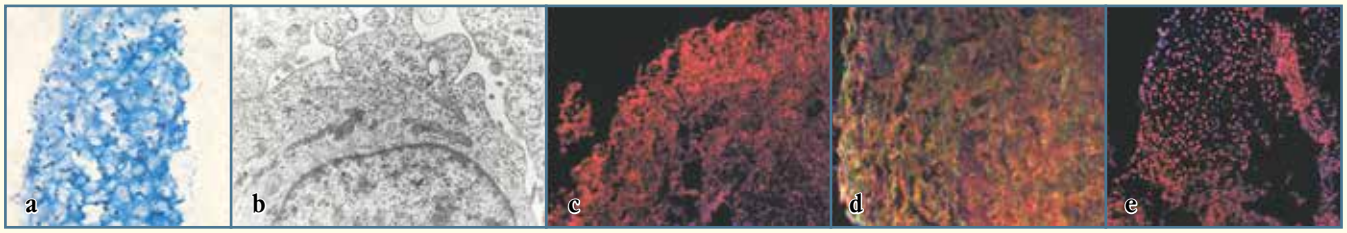

Fig. 1
Chondrograft: **a** – Alcian blue staining, 200; **b** – ultrastructural organization of chondroblast, 5,000; **c** – immunohistochemical test for aggrecan (red); **d** – immunohistochemical test for type I collagen (green) and type II collagen (red); **e** – immunohistochemical test for SOX9 (red)

What should be the process of bone repair? Is it possible to design the intervertebral disc, vertebral body, heel bone, and tibia and to create a bank of spare parts of the musculoskeletal system? Is it possible to produce similar transplant organs composed of autologous cells? Probably, the human mind together with technological progress will create similar grafts, but it is still impossible at present. Why? The cause is the very complex structure and relationships of tissues constituting anatomical structures, e.g., the intervertebral disc with its finest tubules enabling exchange processes with complex metabolism and regulation at the entire body level.

The XXth century is the time of transition from metal mania to cellular technologies whose prospects promise the development of perfect biological grafting materials based on advanced technologies. These are biological designs that are able to restore not only the structural and functional but also homeostatic integrity of bone tissue. Biological cellular technologies have been developed at the Novosibirsk Research Institute of Traumatology and Orthopaedics for many years. There have been successful experiments on the use of cultured chondroblasts for repair of defects in the bone tissue, growth plate, and nucleus pulposus of the intervertebral disc. Cultured chondroblasts were used to develop a chondrograft that was identified at the molecular genetic level and tested as an excellent grafting material. The obtained results were the basis for developing a bone graft in culture medium, which rapidly formed regenerated tissue due to complete integration with the recipient bed and was involved in the recipient's homeostatic system. The stages of these transformations are presented in this lecture.

The chondrograft consists of round cells embedded in a homogeneous Alcian- and Hale-positive matrix (Fig. 1a). Chondroblasts at the graft periphery are predominantly differentiated cells with a large centrally located nucleus, 1 to 2 nucleoli, and heterochromatin. The cytoplasm contains a wide endoplasmic reticulum network, free and attached ribosomes, and a diffusely arranged Golgi complex. Mitochondria are round, with well contoured cristae (Fig. 1b). Chondroblasts express type II collagen, aggrecan, versican, SOX9, biglycan, lumican, etc. (Fig. 1c–e). The structural composition of the chondrograft indicates a high degree of differentiation of chondroblasts.

In culture medium, the osteochondral graft forms in several successive stages, each of which is characterized by morphological, functional, and molecular genotypic features.

Osteoid synthesis stage

After 7 days, the structural composition of the chondrograft changes. Chondrogenic cells undergo partial apoptosis, forming cell-free regions filled with globular eosinophilic masses. Eosinophilic masses are also present in the cytoplasm of cells. In cell-free regions, there are gaps and cavities unrestricted by the endothelial lining (Fig. 2a). Probably, these cavities are a specific microcirculation system similar to that present in definitive and embryonic cartilaginous tissue. The cells are predominantly triangular in shape, with a centrally located nucleus and short processes. The nucleus contains 1 to 2 nucleoli and active (diffuse) chromatin. Eosinophilic masses in the cell cytoplasm and matrix are immunohistochemically identified as sialoproteins (Fig. 2b), the synthesis of which indicates osteogenic differentiation of the cells. The process of chondroblast-preosteoblast-osteoblast differentiation begins at the graft periphery.

Cells with a large nucleus, thin cytoplasm, and contact-forming processes surround the graft. During this period, a structure forms, which may be characterized as a specific barrier or a future periosteum (Fig. 2c). Under the surface layer (presumed periosteum), there are preosteoblasts and type I and II osteoblasts expressing type I collagen and alkaline phosphatase (Fig. 2d, e). It should be noted that, in some areas of the graft, globular protein structures are transformed into a homogeneous pale basophilic matrix. Probably, globular proteins (sialoproteins) associate with type I collagen, and the osteoid is formed, which is the first stage of osteogenic differentiation of the graft. In some areas, the formation of non-mineralized trabecula is observed.

Vascularization stage

After 10–14 days, the chondrogenic graft placed in osteogenic medium is represented by cells at different stages of differentiation – from rare hypertrophic chondroblasts located in

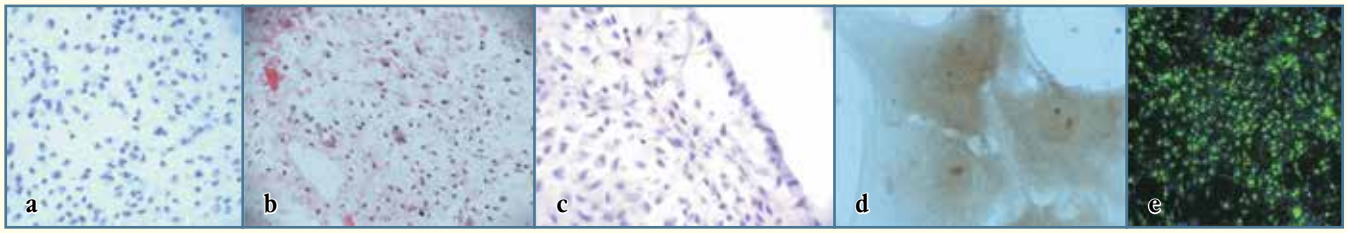

Fig. 2
Osteoid synthesis stage: **a** – cavities without endothelial lining in a bone graft; hematoxylin-eosin staining, 200; **b** – sialoprotein in cells and matrix of the bone graft; hematoxylin-eosin staining, 200; **c** – formation of the periosteum around the bone graft; hematoxylin-eosin staining, 200; **d** – alkaline phosphatase in bone graft cells; 400; **e** – immunohistochemical test for type I collagen (green)

the center to cells with the osteogenic phenotype. These cells contain the CD44 surface antigen, type I collagen, fibronectin, osteonectin, and alkaline phosphatase (Fig. 3b, c). The matrix has a large number of vascular cavities lined with endothelial cells expressing the Willebrand factor and isolectin B4 (Fig. 3d). Around the vessels, there are calcifications identified by Von Kossa staining. The presence of vessels with endothelial lining in the bone graft indicates transformation of the tubular microcirculation system into the vascular system (Fig. 3a).

The question of the nature of endothelial cells in the bone graft remains open. Cell populations of mechanocytes are known to not contain vascular progenitor cells [9]. At the same time, mechanocyte lines form the stroma where descendants of hematopoietic stem cells develop. Bone tissue serves as an essential inducer for differentiation of hematopoietic tissue. The intensity of hematopoiesis depends on the stromal area that is determined by the amount of bone substance. Bone tissue, as a regulator of homeostasis at the organism level, emerges at the earliest stages of ontogenesis. Primary bone tissue serves the basis for development of hematopoietic tissue. It may be supposed that the osteogenesis stages in culture repeat the retained determinacy in formation of bone, vascular, and hematopoietic systems during embryogenesis. Hypertrophic chondrocytes are known to produce the vascular growth factor. According to the literature [5, 12, 13], upon division, hypertrophic chondrocyte undergoes apoptosis, and the second cell adopts the osteogenic phenotype. Further, asymmetric division results in differentiation of the osteogenic cell into osteoblast (endothelioblast). V.G. Gololobov and R.R. Deev adhere to a similar theory [1], calling this cell as a lining, presumably endothelial cell. Is it possible to accept the hypothesis of osteoblast differentiation into the endothelial cell? Why have not the authors identified it? The second explanation is more convincing: differentiation of endothelial cells is the result of synthesis of angiopoietin and cadherin by osteoblasts, which are signaling molecules inducing differentiation or trans-differentiation of osteoblasts into endotheliocytes. The presence of vessels in the bone graft is the metabolic basis for further osteogenic differentiation of the cells – entry into the mineralization stage.

Mineralization stage

The next stage of bone graft formation is the mineralization stage (14–30 days of cultivation). During this period, the cells and matrix undergo transformation – the bone graft becomes a primitive tissue structure. Cells comprising the graft occur at different stages of differentiation, from preosteoblasts to type IV osteoblasts. The latter are cells with a large nucleus and invaginations. The nucleus and organelles are pushed to the membrane by vacuoles surrounded by a bilayer membrane with a light rim. Vacuoles, depending on the degree of maturity, are filled with homogeneous contents of a light to dark color (ultrastructural data). These structures are

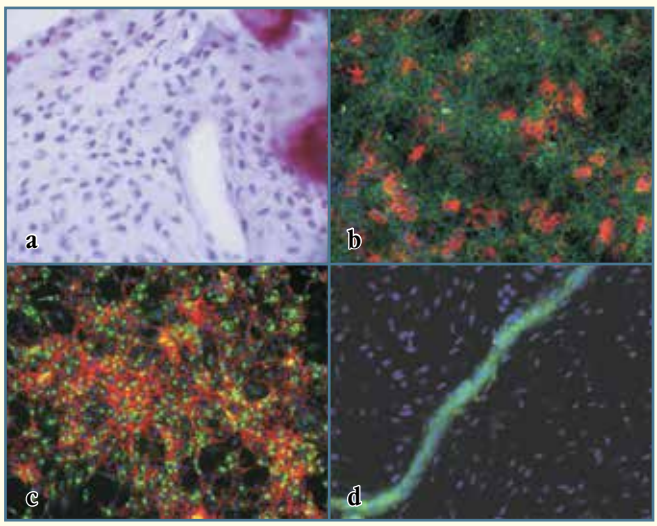

Fig. 3
Vascularization stage: **a** – formed vessels with endothelial lining; hematoxylin-eosin staining, 200; **b** – immunohistochemical test for CD44 (green) and osteonectin (red); **c** – immunohistochemical test for type I collagen (green) and fibronectin (red); **d** – expression of the Willebrand factor in the vascular endothelium of a bone graft

identified as matrix vesicles (Fig. 4a). What are matrix vesicles? These are structures that form on the endoplasmic reticulum and Golgi complex and contain pyrophosphatase, alkaline phosphatase, and inorganic pyrophosphate ($Ca^{++}P$). In matrix vesicles, amorphous pyrophosphate is enzymatically converted to organic hydroxyapatite, dissolution and hydration of crystals occurs, and mineralization centers form. Matrix vesicles are formed from cytoskeleton proteins in type III and IV osteoblasts [4, 6, 10] and are a universal stage of mineralization [11]. In the matrix, alkaline phosphatase-mediated binding of mineral nanocrystals to collagen occurs in the osteoid (Fig. 4d), and the nanocrystals form chains along the collagen fiber; then, ossification takes place. In culture medium, the mineralization stage is limited to the formation of matrix vesicles and their budding and deposition into the matrix (Fig. 4c). Subsequent osteogenesis occurs in vivo after placement of the bone graft into a bone tissue defect.

Bone graft

What is the bone graft? Bone tissue or a bioengineering device? To answer this question, we should consider the bone graft structure. At 14–30 days of cultivation, the bone graft is represented by osteogenic cells, from pre-osteoblasts to type IV osteoblasts. The cytoplasm of the latter is filled with matrix vesicles. Budded vesicles and vessels lined with the endothelium are also localized in the matrix (Fig. 4b). Osteoblasts in the stage I and II of differentiation express the following genes: alkaline phosphatase, osteonectin, osteopontin, type I collagen, transcription factor RUNX2, biglycan, versican, and lumican.

The architectonics of cells in the bone graft is of great interest. Osteogenic cells in the bone graft are arranged in two structural compositions. Densely packed, contacting with each other cells become loose; some cells undergo apoptosis. There is hollow acellular space between cells, which is surrounded by a multilayered ring of osteoblasts. Osteonectin is present in the cavity and cytoplasm of cells. In the second variant, the formed cavity is bounded by two parallel rows of 7 to 10 osteoblasts. In the cavity, the osteonectin protein is found (immunohistochemical data). What are the causes for this arrangement of cells? Is it the two-layer architectonics of osteoblast arrangement? Why? Based on the dynamics of building these structural compositions, it may be supposed that this process reflects the formation of two bone tissue types in ontogenesis: osteon and trabecular bones. We can not assert this idea, but may suggest it. "Modern histology has extensive information on the phylo-ontogenetic development of bone tissue and the metabolism features, but there is a clear gap regarding the initial stages of bone formation". This statement by Friedenstein dates back to 1983, but it still retains its significance at present. Presumably, the development of cellular technologies will help fill this gap in the initial histogenesis of bone tissue.

When answering the question if the bone graft is bone tissue or a bioengineering device (biological equivalent of bone tissue), we should turn to the classical definition of tissue.

Tissue is a system of cells and a non-cellular substance, which is characterized by common phylo-ontogenesis, morphology, and function [3]. The bone graft consists of osteogenic cells expressing bone proteins, genes, and matrix in the initial stage of mineralization. Therefore, the bone graft may be defined as embryonic bone tissue. To confirm this, we should consider repair of a bone tissue defect using a bone graft.

Regenerative potentialities of bone graft in replacement of a vertebral body defect

Bone grafts were transplanted to mini-pigs (6 months old). An anterior retroperitoneal approach to the lumbar vertebral bodies was used under general anesthesia. By using a burr, a bone defect was formed in the anterior part of the vertebral body, with the depth and width corresponding to the size of a graft (about 5 mm). The formed defect was completely filled with the bone graft. Within 14 days, the bone graft placed in the vertebral body defect (Fig. 5a) differentiated into primitive bone tissue of the trabecular structure, including sites where the graft was adjacent to the recipient bed (Fig. 5c). The entire space between newly formed bone structures was filled with osteogenic tissue and blood vessels containing recipient blood cells. This fact indicates integration of regenerated tissue into the recipient's circulation system (experimental animal), which is a factor of histocompatibility of the structures. The formation of a common vascular network and further regulation of osteogenesis in the defect area are controlled by humoral factors supplemented with the recipient's blood at the entire

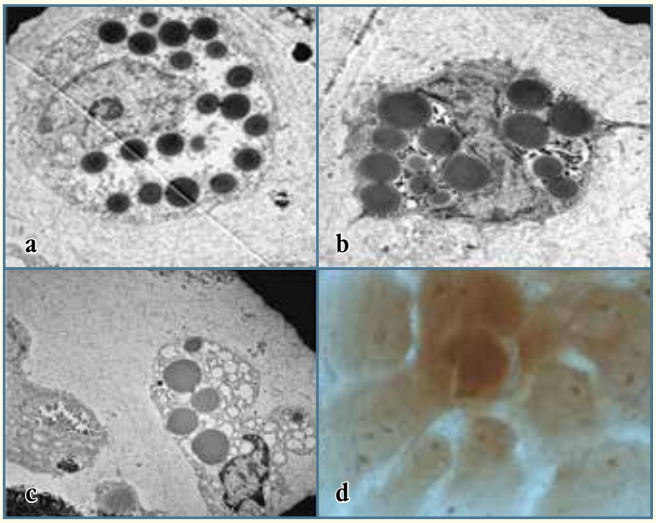

Fig. 4
Mineralization stage. Ultrastructural data **(a–c)**: **a** – matrix vesicles in the cytoplasm of type IV osteoblast; 1,000; **b** – rupture of the osteoblast membrane and release of matrix vesicles; 1,000; **c** – budded matrix vesicles in the bone graft matrix; 1,000; **d** – alkaline phosphatase in cells and matrix of the bone graft; 400

body level (parathyroid hormone, growth factors, signaling systems, etc.). Osteogenic tissue with numerous blood vessels containing erythrocytes spreads from the graft periphery to the intertrabecular spaces of bone tissue in the recipient bed (Fig. 5b). Osteogenesis activation is also observed along the periphery of the bone graft adjacent to the defect margins. The surface of osteocyte-free bone trabeculae is covered by bone tissue represented by the osteoid zone and active osteoblasts, which may be interpreted as complete integration of the graft with the recipient bed. These findings indicate that structural components of the bone graft are responsible for regeneration in the defect area and integration with the recipient bed. After 30 days, the defect cavity is completely replaced by formed trabecular bone tissue (Fig. 5d).

Regeneration of a vertebral body defect and integration with the recipient bed based on the bone graft occur by primary angiogenic osteogenesis within 30 days.

The presence of vessels in the bone graft and their filling with the recipient's blood are the factors of graft integration into the whole system of distant and local regulators of the recipient body.

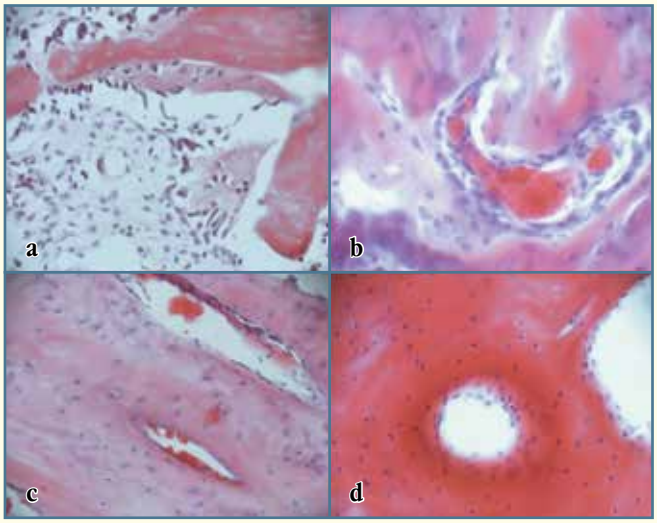

Fig. 5
Regeneration of bone tissue using a bone graft: **a** – the bone graft in a bone tissue defect of the vertebral body; hematoxylin-eosin staining, 200; **b** – graft vessels filled with the recipient's blood; hematoxylin-eosin staining, 200; **c** – after 14 days, the bone tissue defect is filled with primitive bone tissue; hematoxylin-eosin staining, 200; **d** – after 30 days, the defect cavity is replaced by organ-specific bone tissue; hematoxylin-eosin staining, 200

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Received 22.03.2018
Passed for printing 22.04.2018

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