Stimulation of Reparative Osteogenesis after Xenotransplantation of Human Prenatal Mesenchymal Stem Cells and Chondroblasts

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Regeneration of the bone tissue after unilateral xenogeneic transplantation of a suspension of human mesenchymal cells and chondroblasts was studied in rats with experimental damage to both femurs. The state of animals was satisfactory and non-depressed in the early and late postoperation period. No local pathological reactions and complications were seen. Administration of mesenchymal stem cells and chondroblasts into the area of bone defect accelerated regeneration on days 10 and 30 (compared to the control group). Implantation of mesenchymal stem cells more significantly stimulated reparative osteogenesis compared to treatment with chondroblasts. This was mainly associated with increased ratio of mature lamellar bone tissue. Unilateral transplantation of the cell suspension stimulated regeneration in the contralateral limb due to accelerated maturation of the bone tissue. The bone tissue formed after transplantation of mesenchymal stem cells and chondroblasts was integrated into bone organ and underwent complete remodeling. Xenotransplantation of prenatal mesenchymal stem cells and chondroblasts without immunosuppression was not followed by the development of early and delayed complications or local reactions of graft rejection.

Key Words: *reparative osteogenesis; mesenchymal stem cells; chondroblast; histomorphometry*

Bone tissue is characterized by high regeneration activity. However, some traumatic and degenerative disorders of the bone tissue often require treatment with stimulators of reparative osteogenesis [1].

Bone regeneration is based on the interaction between three major biological elements (cells, factors of growth and differentiation, and extracellular bone matrix). Much attention is paid to the search and synthesis of new preparations that can induce/stimulate reparative osteogenesis and modulate activity of these elements. Various methods for stimulation of reparative osteogenesis based on the use of biodegradable biological and synthetic materials were developed and extensively used [4,14]. Another approach suggests treatment with growth and differentiation factors (TGFβ, BMP, FGF, IGF, PDGF, etc.) playing a key role in physiological regeneration of the bone [7,9].

Due to the development of cell biology and possibility to isolate and obtain a considerable number of cultured stem/progenitor cells under laboratory conditions, extensive studies were conducted to evaluate the efficiency of treatment with osteoprogenitor cells to stimulate bone regeneration. This approach holds much promise. Progenitor cells introduced into the

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damaged area secrete various osteogenesis-regulating factors under the influence of microenvironmental factors. They differentiate into osteoblasts and produce substances of the extracellular matrix [10]. Stimulation of bone cell regeneration produces a complex effect on the major pathogenetic stages of bone tissue injury, which can be effectively used in the treatment of diseases of the locomotor system.

Bone tissue regeneration and osteogenesis during embryogenesis involve the same immature mesenchymal cells (preserved in "stem niches" of the periosteum, endosteum, and bone marrow) and are regulated by similar factors of growth and differentiation [2,5,6,9,11]. After adequate reposition and immobilization of bone fragments, mesenchymal progenitor cells (progenitor MC) actively migrate into the site of damage, differentiate into osteoblasts and osteocytes, and determine intramembranous ossification [13]. Under conditions of extensive injury, inadequate reposition of fragments, and insufficient blood supply to the damaged area, MC undergo differentiation into chondroblasts (CB). These changes are accompanied by the formation of temporary cartilaginous tissue. Hypertrophied chondrocytes produce alkaline phosphatase and vascular endothelial growth factor (VEGF), which promotes vascular invasion, migration of progenitor MC for osteoblasts, and mineralization of the extracellular matrix [11,12]. Three populations of self-renewing cells characterized by high proliferative activity and determining the growth and reconstruction of bone tissue (MC, osteoblasts, and CB) can be isolated, cultured, and used for stimulation of regeneration of damaged bone and cartilaginous tissues [1-3,6].

We studied the effect of transplantation of autogenic and allogeneic stem/progenitor cells on reparative osteogenesis. Experimental modeling of bone injury suggests the use of carriers/frameworks for delivery and immobilization of cells in the damaged area. Osteoplastic materials capable of inducing reparative osteogenesis were used as the carriers. Previous studies showed that heterotopic and orthotopic transplantation of the cell suspension was not followed by the formation of normal bone tissue. Bone organ is formed only during transplantation of cells on porous carriers [4]. There is no experimental model to study the stimulatory effect of transplanted cell suspension. The role of transplanted CB in the bone regeneration (*i.e.*, endochondral reparative osteogenesis) is poorly understood.

Here we studied reparative osteogenesis in rat long bones during xenotransplantation of the suspension of human mesenchymal stem cells (MSC) and CB. The effects of cell grafts on the rate and quality of bone tissue regeneration were evaluated.

MATERIALS AND METHODS

Isolation of CB culture. Fresh autopsy specimens were obtained during induced abortion (18-20 weeks pregnancy, 2-3 h postoperation) and used to isolate CB. The women were pre-examined for viral and bacterial infections. Cartilaginous tissue was subjected to mechanical and enzymatic treatment. It was minced and incubated in 0.1% collagenase II (Sigma), 0.05% pronase E (Sigma), and 0.1% hyaluronidase (Sigma) in the 1:1:1 ratio for 90-180 min. Viability of the suspension of individual isolated cells was estimated by trypan blue staining; it was 40-93%. The suspension was placed in 100-mm Petri dishes (Nunc, 2 million cells per 1 ml) with DMEM+F12 growth medium(1:1, Gibco BRL), which contained 10% fetal bovine serum (FBS, PanEko), 1% insulin-transferrin-selenite (PanEko), 50 μg/ml ascorbate (Sigma), 10 μg/ml gentamicin, 5 μg/ml amphotericin B with 5 ng/ml bFGF (Sigma), and 0.1 ng/ml TGFβ$_1$ (Sigma). After attaining a confluent state the cells were subcultured at a density of not less than 1×10^6 cells/ml. Immunophenotypic characteristics of the culture were estimated by means of immunocytochemical staining and flow cytofluorometry. We used monoclonal antibodies to type I and II collagens, aggrecan, CD34, HLA ABC, and DR [3].

ISOLATION OF MSC CULTURE FROM THE BONE MARROW STROMA.

Fresh autopsy specimens were obtained during induced abortion (18-20 weeks pregnancy, 2-3 h postoperation) and used to isolate the culture of bone marrow stromal MSC. The women were pre-examined for viral and bacterial infections. Long bones were separated from soft tissues and placed in a 100-mm plastic Petri dish (Nunc). The bone marrow was aspirated with a syringe containing 3-5 ml Hanks medium. The cells were resuspended in DMEM/F12 growth medium (Life Tech.) with 20% FBS (Hy Clone/Fetal Clone) to a concentration of 100-300 thousands cells per 1 ml; after 24 h nonadherent cells were removed, while adherent cells were washed twice with sterile phosphate buffered saline (pH 7.4). For isolation of the population of rapidly proliferating cells, the culture was seeded at a density of 3 cells/cm^2 in a growth medium containing FGF-2 and 8 U/ml heparin. After 10-12 days the cells were subcultured at a density of 5000 cells/cm^2 and grown to preconfluent monolayer. During the next passage the cell density was decreased to 3 cells/cm^2. After 6-8 days the colonies (colony-forming units, CFU) were taken from dishes pretreated with 1 mM ethylenediaminetetraacetic acid and put to the control dish. The procedure was performed under

an inverted microscope. Then these cells were passed at a density of 50-100 cells/cm^2. No more than 10 passages were performed to obtain grafts. Immunophenotypic characteristics of the culture were estimated by means of flow cytofluorometry using monoclonal antibodies to CD44, CD90, CD105, CD34, HLA ABC, and DR [2].

Preparation of the cell graft. Two-three hours before transplantation, the cultured cells were removed from the plastic with 0.25% trypsin (PanEko) and cell suspension in physiological saline was prepared. The count and viability of cells were estimated by trypan blue staining. Cell count in the graft was brought to 1×10^8 cells/ml. The number of viable cells was not less than 96%.

Transplantation of cells. Experimental damage to rat femoral diaphysis was produced to study the intensity of reparative osteogenesis after xenotransplantation of the MSC and CB suspensions. The experiments were performed on male outbred albino rats weighing 190-200 g. The animals had a typical defect of the femoral diaphysis (diameter 1.5 mm, depth 0.5 mm). The cell graft (1×10^7 cells per 0.1 ml physiological saline) or an equivalent volume of physiological saline was introduced to the bottom of diaphysial defect using an insulin syringe. A polyester sulfonic membrane was applied to fix the graft in the damaged area. The total number of operated rats was 52. Each group included 4-6 animals (for each time point). Each rat had defects in both femurs (the total number of examined objects 84). The animals were sacrificed on days 10, 30, 60, and 90. In treated rats the cell graft was administered into the damaged area in one of the limbs; physiological saline was applied to the contralateral limb. In control animals physiological saline was introduced into the area of defect in both limbs.

We performed 5 experimental series: MSC-experiment (MSC suspension, 1×10^7 cells per 0.1 ml physiological saline); MSC-control (contralateral limb, 0.1 ml physiological saline); CB-experiment (CB, 1×10^7 cells per 0.1 ml physiological saline); CB-control (contralateral limb, 0.1 ml physiological saline); and control group (0.1 ml physiological saline, both limbs).

Apart from monitoring of the general state of animals and experimental wounds and macroscopic examination during autopsy, we carried out a histomorphological study of treated tissues and histomorphometric analysis of changes in tissue components of the bone regenerate.

Histological study and morphometry. Bone objects were isolated, fixed with 10% neutral formalin,

TABLE 1. Morphometric Indexes in Groups (%, $M\pm m$)

Period, days	Experimental series	Bone part of regenerate	Bone matrix	Mature bone matrix	Connective tissue	Hemopoietic tissue
10	MSC-experiment	98.4±4.7*+o	72.96±8.00*+o	0.82±1.80	2.2±2.0*+o	28.1±14.5+
	MSC-control	88.4±4.7*	58.9±20.2	0	11.2±5.8*	18.4±6.8*
	CB-experiment	94.5±3.4*+	62.2±8.9	0	6.2±3.1*+	0
	CB-control	85.98±5.20*	60.4±5.7	0	13.80±6.98*	0
	Control	80.1±2.3	64.4±6.9	0	21.30±2.01	11.7±6.9
30	MSC-experiment	100*+o	81.5±8.7*+o	24.70±5.03*+	0	5.5±2.8*o
	MSC-control	89.8±5.7*	77.6±7.1	15.4±5.9*	0	0.52±1.30*
	CB-experiment	97.5±2.1*+	79.5±13.1*+	16.3±5.4*	2.70±2.37*	16.3±1.5
	CB-control	93.5±6.2*	71.2±19.1	16.0±13.9*	7.4±6.4*	17.9±9.9
	Control	83.2±4.3	75.40±8.85	10.70±4.85	16.1±8.6	13.70±5.16
60	MSC-experiment	98.2±2.4	90.2±3.7*+o	23.4±13.9*+o	0	2.9±3.4
	MSC-control	96.3±5.3	75.7±12.5	17.9±5.7	2.8±3.2	8.9±8.7
	CB-experiment	100	90.97±9.10*+	36.1±15.7*+	0	2.3±2.6
	CB-control	98.4±1.1	83.6±9.9	18.5±7.5	2.5±1.9	5.7±5.2
	Control	96.4±4.5	84.5±4.9	21.5±9.6	3.0±4.1	9.6±6.2
90	MSC-experiment	100	89.70±8.95	56.7±11.8	0	0
	MSC-control	94.1±6.2	98.7±3.3	51.2±18.2	3.2±4.8	2.9±7.6
	CB-experiment	100	98.8±2.0	51.1±19.8	0	0
	CB-control	97.2±3.5	98.7±5.0	53.6±5.1	2.3±2.9	0.57±1.20
	Control	100	93.5±4.5	49.9±12.7	0	2.6±3.0

Note. $p<0.05$: *compared to the control; +compared to MSC-control and CB-control, respectively; ocompared to CB-experiment.

and decalcified in 25% Trilon B. Bone marrow fragments were routinely embedded into paraffin. Serial sections through the thickness of the examined objects were prepared. The sections were stained with hematoxylin and eosin, subjected to Van Gieson staining, and silver-impregnated by the method of Foot.

Stained sections were photographed. We measured the area of the regenerate, bone component of the regenerate, bone matrix, connective tissue, and hemopoietic tissue. The area of structural components in the regenerate was compared with the total area of the regenerate. These relative values were processed statistically.

RESULTS

During the early and late postoperation period, the state of animals was satisfactory and non-depressed. There were no local pathological reactions and complications. We revealed no rejection reaction, inflammation, or lymphohistiocytic infiltration at the site of xenogeneic transplantation.

Day 10 of the experiment. Regenerate consisting of connective tissue and bone components was formed in animals of various groups after 10 days. The specific weight of each component strongly correlated with experimental conditions. In the control group, the fibrocellular connective tissue lay between edges of the bone defect. Immature bone osteoid and fibrous trabeculae were found only in deep layers of the bone wound (level of the bony canal, Fig. 1). In this period newly formed bone structures appeared in animals of the main and reference group. They replaced the connective tissue component of the regenerate between fragments of the cortical plate (Fig. 2, *a-d*). The connective tissue looked like a narrow fibrous band between bone structures. Only in the CB-control group the connective tissue constituted a considerable part of the regenerate (similarly to control animals). Immature bone structures were found only at the periphery of the bone defect (Fig. 3, *b*).

Intergroup differences were found in the degree of differentiation of structural components in the regenerate. In animals of MSC-experiment and MSC-con-

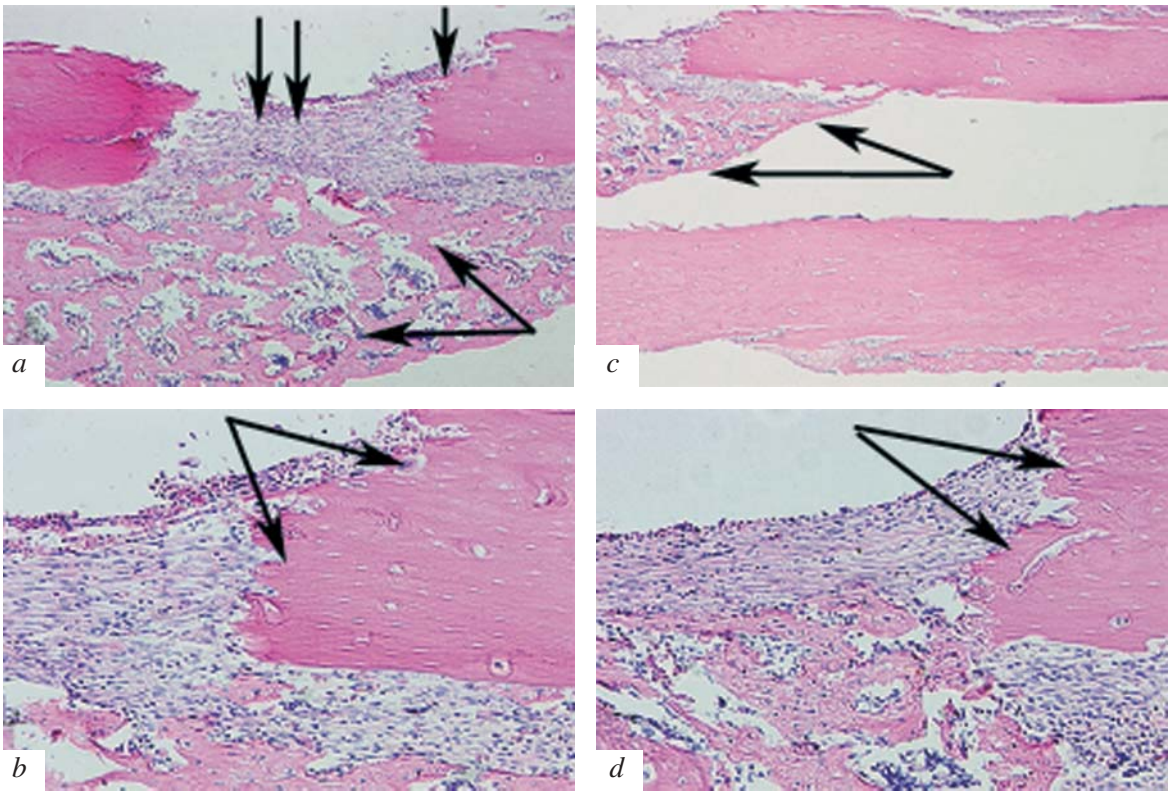

Fig. 1. Normal reparative osteogenesis in rat on day 10. Staining with hematoxylin and eosin. *a*) bone defect involving the total width of the cortical layer is filled with fibrocellular connective tissue. Narrow band-like infiltrate consisting of leukocytes, lymphocytes, and macrophages on the surface (arrow); eroded edges of cortical plate fragments. Absence of osteocytes over a considerable length of the bone matrix. Large bone regenerate formed by a narrow-loop reticulum of thin newly formed bone trabeculae and located in the bone defect under connective tissue (level of the bony canal, arrows at an angle, ×25); *b*) eroded edge of bone fragments; giant multinucleated osteoclasts in resorption lacunas (arrows). Absence of osteocytes in the bone matrix (bone edge of defect, ×200); *c*) a net of newly formed bone trabeculae (arrows) and a connective tissue bundle run under the cortical plate and enter the bony canal. Minor periosteal reactions in the area of trauma, ×25; *d*) lower surface of the cortical plate adjacent to fibrocellular tissue in the bony canal and characterized by slight usuration (2nd observation); individual resorption lacunas (arrows, ×200).

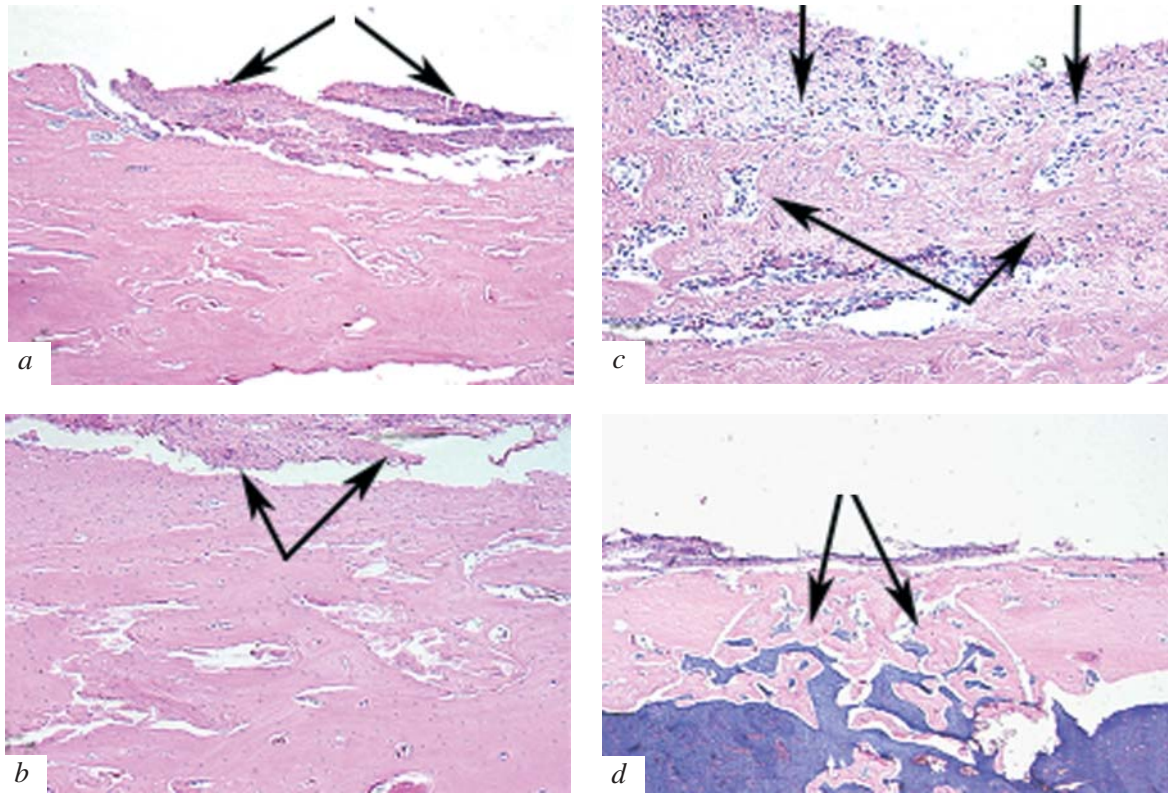

Fig. 2. Reparative osteogenesis in a rat 10 days after transplantation of human MSC. Hematoxylin and eosin staining. *a*) connective tissue part of the regenerate looks like a narrow band of the fibrocellular tissue at the level of the upper surface of the cortical plate (arrows). Bone regenerate occupies the lower part of the cortical plate. Structures of the regenerate undergo maturation and compaction; the bone matrix somewhere gain a lamellar structure, ×50; *b*) surface localization of the connective tissue layer (arrows); newly formed bone part of the regenerate is located under the connective tissue layer and undergoes compaction, ×50; *c*, *d*) MSC-control group; *c*) surface narrow band of fibrocellular and partially fibrous connective tissue (arrows). Trabeculae of dense-fibrous substance in the internal space (arrows at an angle, ×200); *d*) newly formed bone structures between cortical layer fragments (arrows, ×25).

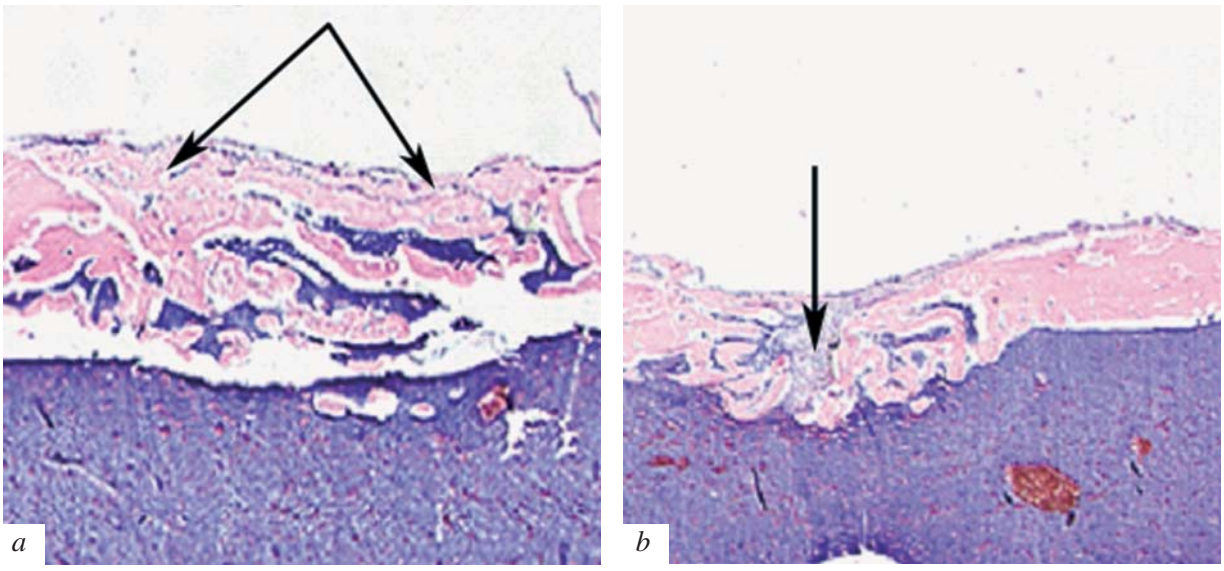

Fig. 3. Reparative osteogenesis in a rat 10 days after transplantation of human CB. Hematoxylin and eosin staining. *a*) CB-experiment group. Bone regenerate is formed by a narrow-loop reticulum of immature bone trabeculae and located in the bone defect at the level of the cortical plate (arrows, ×35); *b*) CB-control group; *c*) extensive zones of connective tissue (arrow) and well-developed newly formed bone structures in the regenerate, ×20.

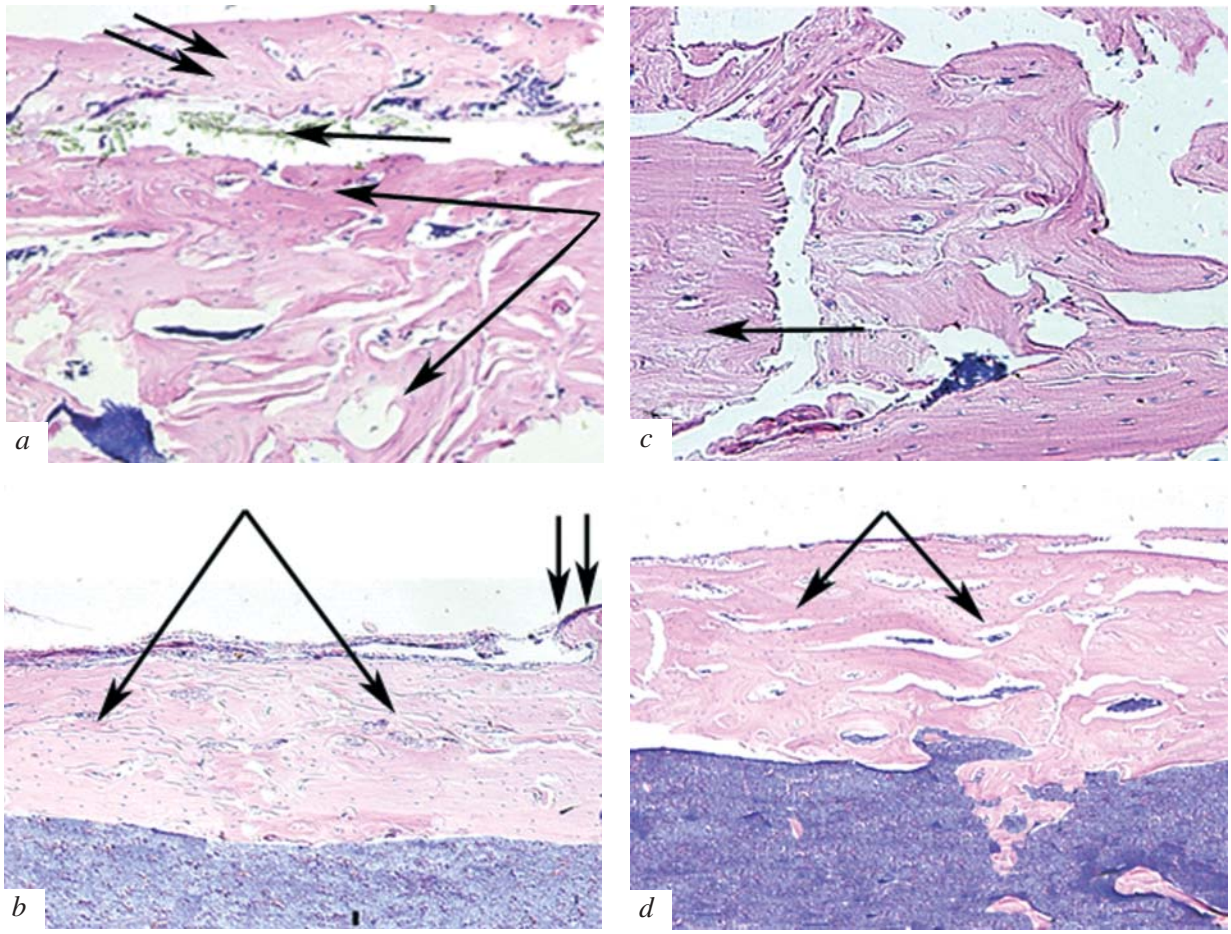

Fig. 4. Reparative osteogenesis in a rat 30 days after transplantation of human MSC and CB. Hematoxylin and eosin staining. (*a*) MSC-experiment group. Newly formed mature bone tissue in the bone defect (arrows at an angle). Polyester sulfonic membrane covers the bone regenerate (arrow). Periosteal bone strata over the membrane (double arrows, ×70); *b*) MSC-control group. Well-structured bone regenerate is located in the bone defect (arrows at an angle) and undergoes compaction. Small residual intertrabecular spaces filled with islets of loosened connective tissue. Polyester sulfonic membrane covered with fibrous connective tissue; bone matrix outgrowths at the edges of the membrane (double arrows, ×70); *c*) CB-experiment group. Fusion of the bone regenerate and maternal bone fragment (arrow). Newly formed bone matrix is structurally similar to the cortical plate matrix. Signs of tissue sclerosis in intertrabecular spaces, ×280; *d*) CB-control group. Bone regenerate completely fills the spaces between fragments of the cortical layer in maternal bone and has a cancellous structure (arrows). Groups of newly formed bone trabeculae embedded into the bone marrow, ×70.

trol groups intensive formation of bone structures and maturation of the bone matrix were noted (Fig. 2, *a*, *b*). The matrix had fibrous structure at considerable length, but somewhere contained small fragments with lamellar structure. No lamellar structures were found in control animals.

The regenerate mainly consisted of bone components on day 10 after administration of CB into the bone wound. The newly formed bone tissue had irregular structure and was characterized by a lower degree of cell differentiation compared to animals receiving MSC.

The existence of intergroup differences was confirmed by the results of histomorphometric analysis. We studied the development of major components in the regenerate. The area of the bone components increased significantly, while the ratio of the connective tissue decreased in animals receiving MSC. The degree of bone matrix maturation increased in these rats. In animals of the MSC-experiment group, the ratio of bone components in the regenerate increased compared to that observed in the bone wound of the contralateral limb (MSC-control). The intensity of bone regeneration in animals of the MSC-experiment group was higher than in the CB-experiment group. Ten days after administration of CB into the bone wound, the ratio of bone components in the regenerate was much higher compared to animals of the control and CB-control groups.

These results show that transplantation of MSC and CB into the bone wound stimulates reparative osteogenesis. The intensity of this process was higher in animals receiving MSC compared to rats of the CB-experiment group. Moreover, the ratio of bone components in rats of the MSC-control and CB-con-

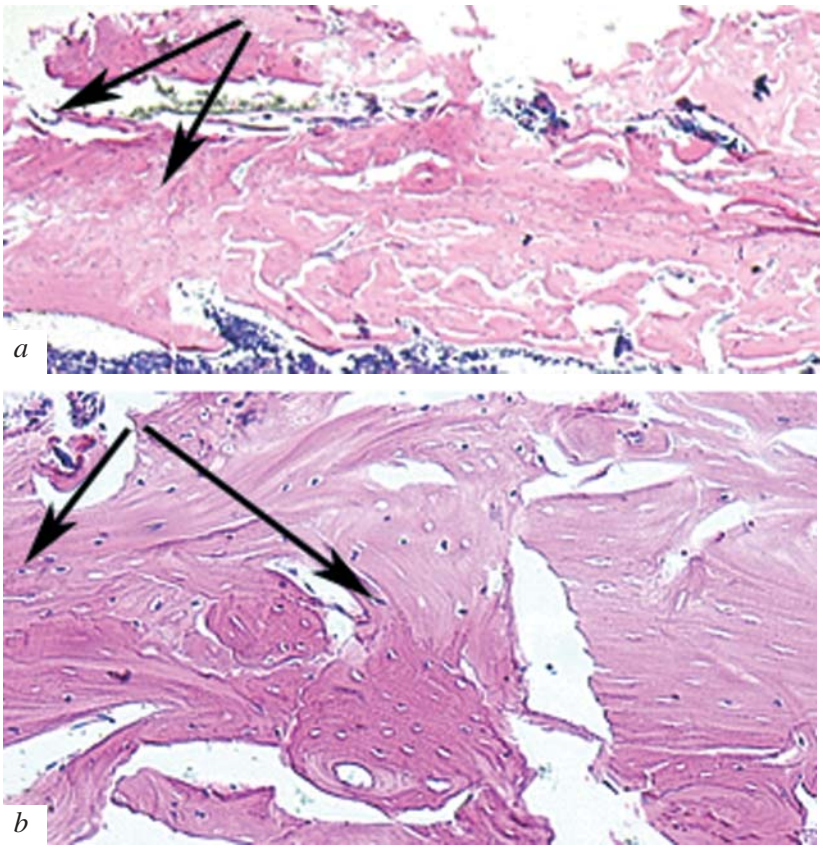

Fig. 5. Reparative osteogenesis in a rat 60 days after transplantation of human MSC and CB. Hematoxylin and eosin staining. *a*) MSC-experiment group. Bone regenerate between fragments of the cortical plate is presented by cancellous structures, somewhere with a tendency to compaction (arrows, ×50). *b*) CB-experiment group; compacting differentiated bone tissue of the regenerate (arrows, ×100).

trol group was much higher than in control animals (Table 1).

Day 30 of the experiment. Bone defect in the cortical plate of treated animals was completely filled with the bone regenerate. Examination of some histological preparations from control animals revealed connective tissue islets with a considerable number of cells in the outer part of the regenerate. The space between maternal bone fragments was filled with a net of trabeculae composed of fibrous and lamellar substance. Polyester sulfonic membranes covering the bone defect were embedded in the newly formed bone tissue (Fig. 4, *a*). After administration of MSC the regenerate in both limbs was formed by large trabeculae with narrow intertrabecular spaces. Its bone matrix underwent active differentiation, was characterized by high density (similar to the maternal bone), and had lamellar structure. In animals receiving CB the bone defect was completely replaced with cancellous bone regenerate (Fig. 4, *c*, *d*). Bone trabeculae in the regenerate consisted of a dense matrix, which practically did not differ from the matrix of adjacent cortical plate fragments. The cortical plate had a cancellous structure, which was related to secondary reconstruction.

Histomorphometric analysis showed that on day 30 of the experiments, the regenerate in control and treated rats was formed by bone structures. Connective tissue regions were present in regenerates of control animals. On day 30 we revealed more active compaction (increase in bone matrix ratio) and differentiation of the bone matrix, particularly in the MSC-experiment group (Table 1).

Day 60 of the experiment. Further development and differentiation of bone structures in the regenerate were seen in the control and experimental groups. In control animals the bone regenerate had a cancellous structure. The extracellular matrix included dense and lamellar regions. A similar tendency towards maturation of the bone regenerate was found in experiments with administration of cell grafts into the bone wound (Fig. 5). No significant intergroup differences were revealed in this period (Table 1).

Day 90 of the experiment. Histological study showed that the intensity of reparative osteogenesis and maturation of the bone regenerate remained high in controls and animals receiving cell transplants. We found no intergroup differences in histomorphometric characteristics of structural components in the regenerate (Table 1).

The proposed experimental model of bone injury allowed us to study reparative osteogenesis after treatment with the cell suspension graft. Properly fixed polyester sulfonic membrane remained in the area of implantation until the end of the experiment. It prevented migration of periosteal elements to the bone defect and affected the course of reparative processes, which mainly involved extraperiosteal cells (cells of the bone defect).

Administration of cells grafts from MSC and CB produced a stimulatory effect on reparative osteogenesis, which was most pronounced on day 10 of the experiment. After unilateral administration of the cell graft, this effect was revealed in both limbs. However, the degree of changes varied in the treated and contralateral limbs. The appearance of bone trabeculae and maturation of the bone tissue in the regenerate were found in the delayed period after treatment. On day 90 the bone regenerate completely developed in the area of defect in animals of different groups. The newly formed bone tissue was integrated into bone organ. Xenotransplantation of prenatal MSC and CB without immunosuppression was not followed by the development of early and delayed complications or local reactions of graft rejection. These data substantiate the therapy of extensive bone damage with allogeneic implantation of prenatal MSC and CB.

REFERENCES

1. A. K. Dulaev, V. G. Gololobov, R. V. Deev, *et al*., *Med. Akadem. Zh*., **3**, No. 3, 59-66 (2003).
2. A. A. Rzhaninova, S. N. Gornostaeva, and D. V. Gol'dshtein, *Klet. Tekhnol. Biol. Med.*, No. 1, 34-41 (2005).
3. T. Kh. Fatkhudinov, D. V. Gol'dshtein, V. M. Govorun, *et al*., *Byull. Eksp. Biol. Med.*, Suppl., 44-52 (2003).
4. K. Anselme, B. Noel, B. Flautre, *et al*., *Bone*, **25**, No. 2, 51S-54S (1999).
5. R. Cancedda, F. Descalzi-Cancedda, and P. Castagnola, *Int. Rev. Cytol*., **159**, 265-358 (1995).
6. A. J. Friedenstein, R. K. Chailakhyan, and U. V. Gerasimov, *Cell Tiss Kinet.*, **20**, No. 3, 263-272 (1987).
7. L. C. Gerstenfeld, D. M. Cullinane, G. L. Barnes, *et al*., *J. Cell. Biochem.*, **88**, 873-884 (2003).
8. M. Kassem, *Cloning Stem Cells*, **6**, No. 4, 369-374 (2004).
9. J. R. Lieberman, A. Daluiski, and T. A. Einhorn, *J. Bone Joint Surg. Am.*, **84-A**, No. 6, 1032-1044 (2002).
10. H. Petite, V. Viateau, W. Bensaid, *et al*., *Nat. Biotechnol*., **18**, No. 9, 959-963 (2000).
11. H. Qi, D. J. Aguiar, S. M. Williams, *et al*., *Proc. Natl. Acad. Sci. USA*, **100**, No. 6, 3305-3310 (2003).
12. H. J. Roach, J. Erenpreisa, and T. Aigner, *J. Cell. Biol*., **131**, No. 2, 483-494 (1995).
13. J. U. Yoo and B. Johnstone, *Clin. Orthop*., **355**, Suppl. 73-81 (1998).
14. J. Y. Zhang, B. A. Doll, E. J. Beckman, and J. O. Hollinger, *Tiss. Eng*., **9**, No. 6, 1143-1157 (2003).