Effect of Mesenchymal Stem Cells on Rejection of Xenogenic Bone Transplant

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We studied the effects of mesenchymal stem cells on rejection of xenogenic bone transplant. Experiments were carried out on Wistar—Kyoto rats transplanted chicken demineralized bone matrix populated or not populated with mesenchymal stem cells to the site of parietal bone defect. Histological analysis showed complete resorption of the xenogenic transplant not populated with mesenchymal stem cells by day 119. In experimental animals chicken matrix without signs of tissue inflammation was in fact completely retained over the entire period of observation. Numerous new vessels, mineralization fields, and areas of bone tissue formation were seen in the transplant.

Key Words: *mesenchymal stem cells; xenogenic bone transplant; immunosuppression*

The total systemic immunomodulating effects of mesenchymal stem cells (MSC) after allogenic or xenogenic transplantation are now intensively studied [2,6,14]. The first positive results of clinical application of transplantation of allogenic hematopoietic cells together with MSC are reported [11,12].

MSC are pluripotent stem cells, originating mainly from the red bone marrow [10] and capable of differentiation in the osteogenic, chondrogenic, adipocytic (so-called orthodox directions), myocytic, cardiomyocytic, and neuronal directions [5,7, 8,13]. It is assumed that MSC do not carry CD34, CD45, CD14, glycophorin A, T- or B-cell markers but carry Thy-1 (CD90), VCAM-1 (CD106), endoglin (CD105), and hyaluronic acid receptor (CD44) on their surface [9].

It has been experimentally proven that MSC modify the course of tissue inflammation [1], activate angiogenesis [3], and improve cell survival in tissue zones adjacent to the defect [4].

Here we studied the effect of MSC on the rejection of xenogenic bone transplant.

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MATERIALS AND METHODS

Experiments were carried out on inbred male Wistar—Kyoto rats (180-200 g).

Bone marrow suspension was isolated from femoral bones directly after decapitation. The femoral bone epiphyses were removed under sterile conditions, the diaphyses were washed with αMEM (HyClone) supplemented with 20% fetal calf serum (Gibco) and 100 μg/ml penicillin or streptomycin (Gibco). The suspension was inoculated in plastic Petri dishes (Sarstedt). Forty-eight hours after bone marrow explantation MSC were twice washed from blood cells in PBS (20 mM PBS, pH 7.4; 0.1 M NaCl). The cells were cultured in a monolayer at 37°C and 5% CO_2 for 6-7 days after explantation, after which the culture was reinoculated every 7 days at the initial cell density of 1.27×10^3 cell/cm^2. Trypsin and EDTA (HyClone) were used for reinoculation of rat MSC. Nutrient medium was replaced every 3 days.

Rat MSC were phenotyped on a FACSscan cytofluorometer (Becton Dickinson). MSC were stained with antibodies to CD45 negative marker (Becton Dickinson) and antibodies to CD90 positive marker

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(Becton Dickinson). To this end, the cells were removed from dishes with trypsin and EDTA (HyClone), washed twice in PBS, transferred for 1 h into solution (1:20) of fluorochrome-conjugated monoclonal antibodies, after which they washed twice in PBS and fluorescence intensity was evaluated. Phenotyping was carried out after the first, second, and third reinoculation of the culture.

For more complete characterization of the obtained cell material, the cell capacity to differentiate in the chondrogenic, osteogenic, and adipocytic directions was verified, phenotyping and morphological analyses of the derivatives were carried out. Phenotyping was carried out by the analysis of marker gene expression by polymerase chain reaction with reverse transcription [1].

Xenogenic demineralized bone matrix populated or not populated with MSC was used for transplantation. Bone matrix was prepared from flat parietal bones of chicken skull. Bone plates were placed in 0.5 M hydrochloric acid for 3 days, acid solution was replaced 3 times. The plates were washed in distilled water and plunged in diethyl ester for 1-2 h. The bone matrix was then washed 5-7 times in water and plunged in 70% ethanol, after which demineralized bone matrix was put into MSC culture medium for 12 h (the medium was replaced twice). MSC suspension was applied onto the matrix (2.5 million cells per bone plate), which was put into PBS for 48 h. The efficiency of the method was verified as follows: MSC were stained with PKH26 fluorochrome, applied onto the matrix, and after 7 days fluorescent microscopy (Zeiss-Axioscope) confirmed complete population of the matrix with live MSC (Fig. 1).

Ready sterile bone matrix was transferred to the site of bone injury in experimental animals. The rats were narcotized with sodium pentobarbital (40 mg/kg intraperitoneally). An 8-mm hole was drilled with a dental drill in the parietal region of the skull without damaging the dura mater. The transplant (chicken demineralized bone matrix populated with MSC or without MSC) was applied onto the defect and the wound was sutured. The site of the defect was visually examined on days 45, 56, 65, and 119 after transplantation and the skulls of 2-5 rats from experimental and control groups per term were X-rayed, after which the animals were decapitated and histological analysis of tissues formed at the site of injury was carried out.

The parietal bone fragment, including the site of injury, implanted matrix, and adjacent tissues, was dissected directly after decapitation. Bone tissue fragments were fixed in 8% formalin for at least 3 days. After fixation the bone plates were demineralized with EDTA. The preparations were then embedded in paraffin by the standard method and 7-μ sections were made. The preparations were routinely stained with hematoxylin and eosin.

RESULTS

By day 45 after transplantation of the xenogenic matrix without MSC severe leukocytic inflammation was observed at the site of defect (Fig. 2, *a*). The structure of the chicken matrix was preserved, but resorption has started. No new bone tissue cells were detected in the implant. By day 56 the lamellar structure of the matrix was lost. Large spaces between the bars were filled with the connective tissue. No osteocyte-like cells were detected in the lacunae. Connective tissue in the spaces between the bars was well vascularized, but no signs of its mineralization were observed. By day 65 the chic-

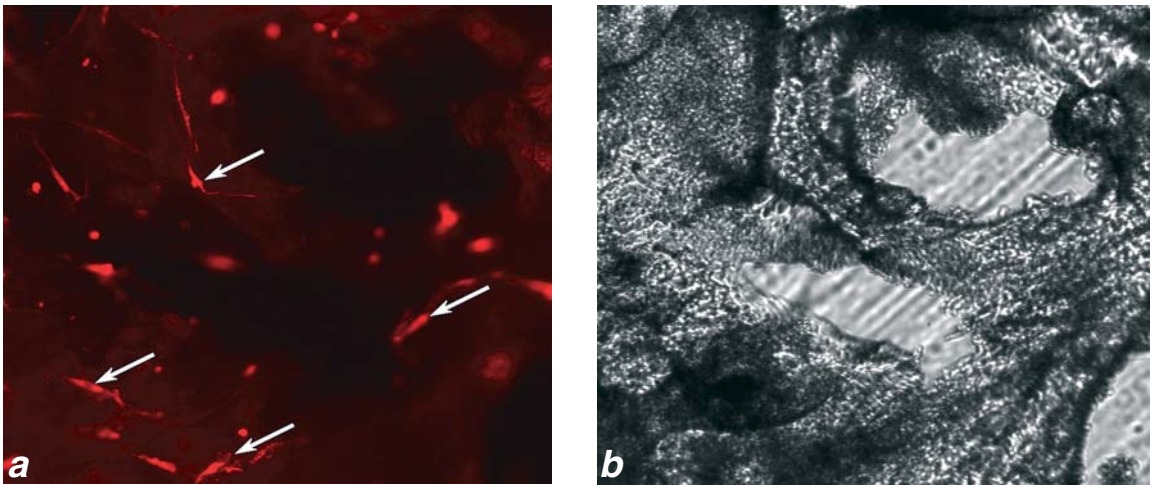

Fig. 1. Demineralized chicken bone matrix populated with rat MSC (×20). *a*) visualization of MSC fluorescent staining; *b*) phase contrast. Arrows show MSC.

ken matrix was considerably resorbed and replaced with loose connective tissue with numerous vessels, but without mineralization and population of the lacunae with osteogenic cells (Fig. 2, *b*). By day 119 the xenogenic matrix was virtually completely resorbed. Compact connective tissue with few osteocytes formed between the small retained areas of the matrix (Fig. 2, *c*). The material covering the site of the defect differed from the intact bone by X-ray density (Fig. 3, *a*).

Virtually no inflammation was seen at the site of the defect after transplantation of xenogenic

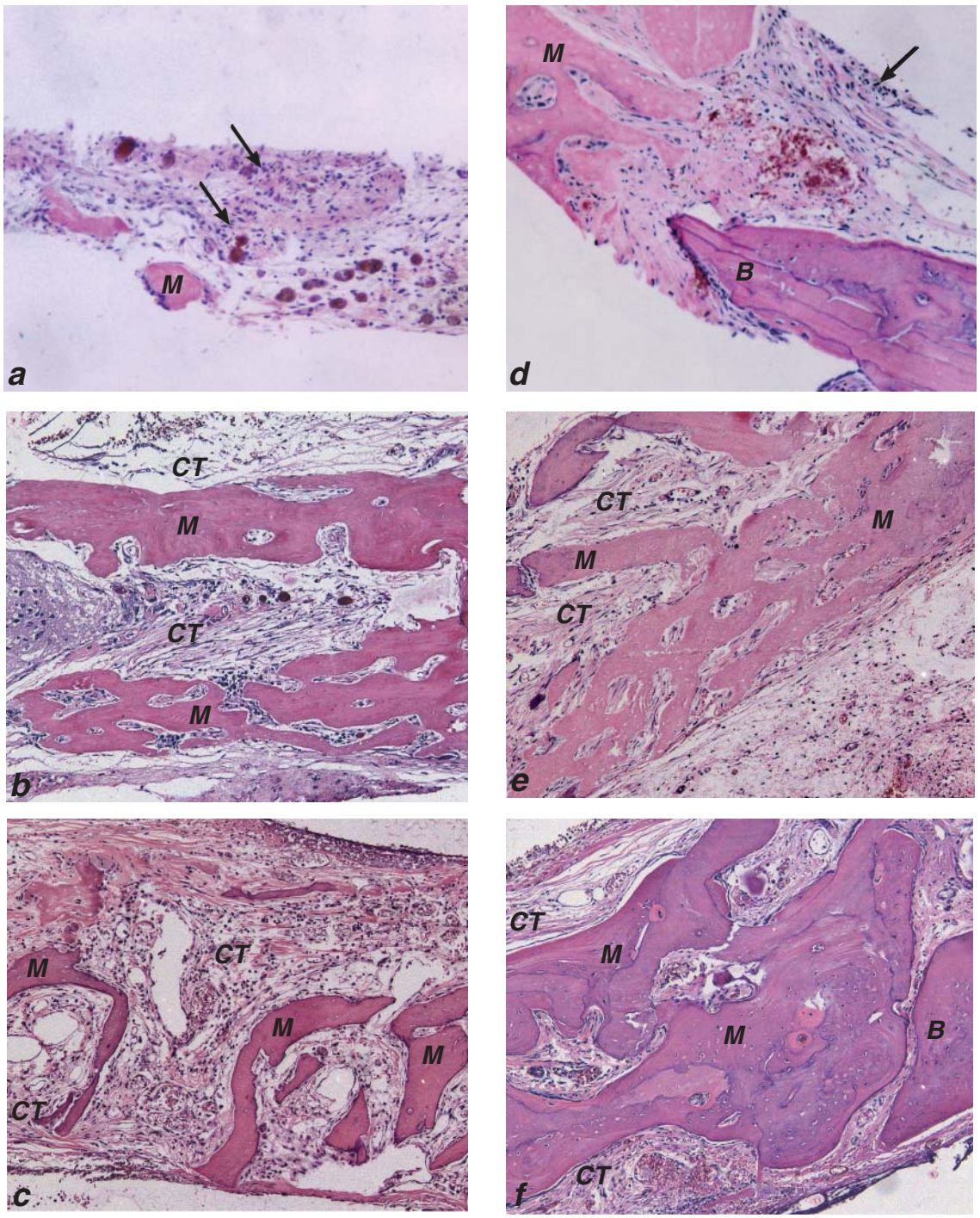

Fig. 2. Chicken bone matrix (*a-c*) and chicken bone matrix populated with rat MSC (*d-f*) 45 (*a*, *d*), 65 (*b*, *e*), and 119 days (*c*, *f*) after transplantation to the rat parietal area. *M*: matrix; *CT*: connective tissue; *B*: recipient bone. Arrows show the focus of inflammation. Hematoxylin and eosin staining (×35).

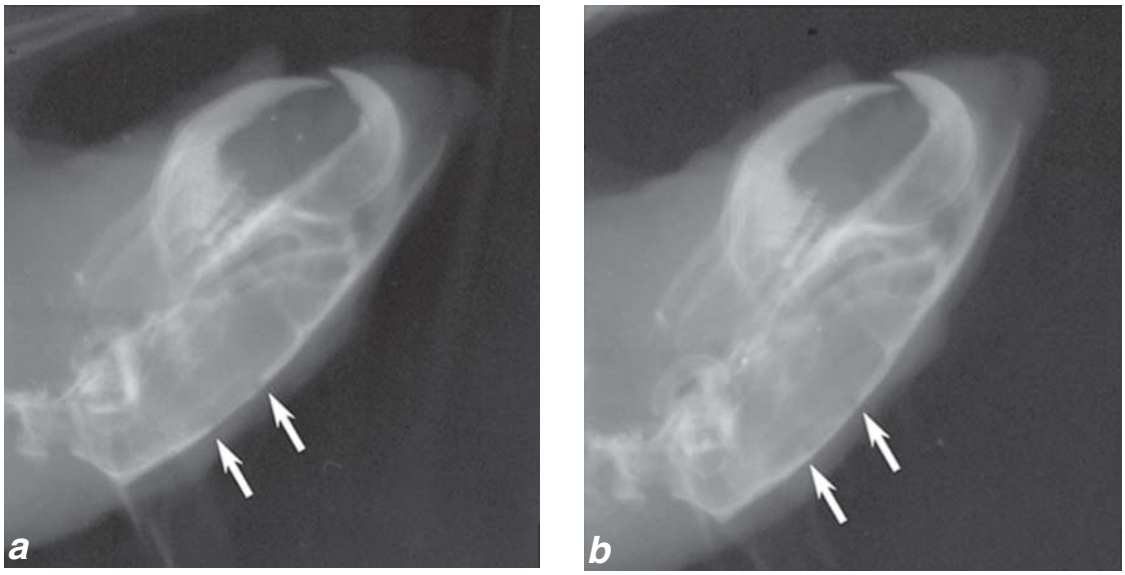

Fig. 3. Roentgenograms of rat skulls 119 days after transplantation of chicken matrix (*a*) or chicken matrix populated with rat MSC (*b*). Lateral view. Arrows show the interface between the transplant and recipient bone.

matrix populated with syngeneic MSC (Fig. 2, *d*). Negligible resorption was observed only at the transplant periphery. Osteocytes in the lacunae and numerous vessels in the connective tissue between the bars were seen. By day 56 the lamellar structure of the matrix was somewhat impaired, spaces between the bars were enlarged. Numerous vessels and areas of mineralization were seen in the transplant. The integrity of transplanted matrix was retained for 65 days. Spaces between the bars were filled with the connective tissue with numerous vessels. Osteocytes were seen on the bar surface (Fig. 2, *e*). By day 119 the xenogenic matrix populated with MSC was in fact completely preserved. Numerous vessels were seen in the transplant. Neoosteogenesis foci were detected in the peripheral lacunae. The matrix was slightly mineralized (Fig. 2, *f*). The site of the defect was clearly seen on the roentgenograms until day 65: it was covered by tissue of lower X-ray density than the bone. After 119 days the transplant was difficult to differentiate from the intact bone (Fig. 3, *b*).

Hence, the results indicate that application of MSC onto xenogenic bone matrix preserved its integrity at the site of transplantation without signs of tissue inflammation. MSC were viable and differentiated in the osteogenic direction. An appreciable number of new vessels prevented ischemia of the transplant and promoted further formation of the bone tissue. Presumably, MSC prevented the graft versus host reaction. Our data are in good agreement with the results of clinical studies in which MSC inhibited the graft versus host reaction [11,12].

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