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Improving Biocompatibility of Heart Valve and Vascular Transplants by Their Devitalization and Repopulation by Recipient Cells

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Inoculation of cells derived from the aorta of Wistar rats on devitalized porcine aortic walls 2-4-fold reduced their calcinosis after subcutaneous implantation to Wistar rats. Inoculation of Wistar rat bone marrow mesenchymal cells selected by adhesion activity did not reduce tissue calcinosis. The results indicate good prospects of repopulation of devitalized heart valve and vessel transplants by recipient vascular cells for reducing transplant calcinosis and improvement of their biocompatibility.

Key Words: *heart valves; vessels; devitalization; cell repopulation; calcification*

Destruction of donor cells (devitalization) and subsequent repopulation of heart valve and vessel transplants by recipient cells is regarded as a perspective approaches to the improvemnt of their biocompatibility and prolongation of their functioning [1,2,5-8,10-15]. Destruction of donor cells is expected to reduce the graft immune response and prevent calcinosis, while repopulation with recipient cells will stimulate repopulation and recovery of native matrix structure. It is not yet quite clear how justified these expectations are. We tried to evaluate to what degree repopulation of devitalized transplants by recipient cells could prevent calcinosis. We used an experimental approach including devitalization of porcine aortic cells, preparation of cell culture from the aorta and bone marrow of Wistar rats, inoculation of cells on devitalized aortic walls, and their implantation to Wistar rats.

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MATERIALS AND METHODS

Smooth-muscle cell culture (SMC) was derived from Wistar rat aorta as described previously [1]. The cytoskeleton of isolated cells bound FITC-conjugated monoclonal antibodies to SMC α-actin (Sigma). These cells did not bind monoclonal antibodies to von Willebrand factor or ulex Europaeus lectin (endothelial cell markers).

Bone marrow mesenchymal cells were isolated from the femoral bones of Wistar rats [4]. Fraction of cells selected by adhesion to culture flasks (1-h incubation in DMEM with 10% FCS) was used. These cells also bound antibodies to SMC α-actin and did not react with the above markers of endothelial cells. Cells isolated from the vessels and bone marrow were cultured in DMEM (Sigma) with 10% FCS (HiClone) and 50 μg/ml gentamicin at 37°C and 5% CO_2. The cells of passages 1-5 were used (culturing until 15 doubling of cell count, the Hayflick limit is about 30 doublings).

Porcine aortic heart valves were removed no later than 1 h after sacrifice and placed into RPMI 1640 with gentamicin (400 μg/ml) and fluconazole

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(50 μg/ml). Tissue devitalization was started no later than after 4 h using multistep enzyme treatment [6,15], which, according to our data, did not prevent tissue calcification [2]. Enzyme treatment [15] was modified by digitonin (0.01%) addition to trypsin and EDTA solution. Devitalization of tissues was carried out for 24 h in DMEM (Sigma) with 0.01% digitonin which binds to cholesterol thus forming pores in plasma membrane, causing cell death. All stages of the treatment were performed at 37°C. After treatment the tissues were washed in RPMI 1640 for 24 h in order to remove damaging agents.

Express analysis of cell viability in aortic wall tissues and aortic valve leaflets was carried out by fluorescent microscopy [1]. A tissue fragment was incubated for 10 min at 37°C in DMEM with 5 μg/ml ethidium bromide (staining only nuclei in dead cells, red fluorescence) and 5 μg/ml Hoechst 33342 bisbenzimide (staining the nuclei of live and dead cells, blue-green fluorescence) and the counts of live (blue-green fluorescence of the nuclei) and dead (orange fluorescence) cells was evaluated by fluorescent microscopy. The method can be used for detecting and evaluating viability of endothelial cells on tissue surface, SMC and fibroblasts in the tissue, mitotic activity and apoptotic cell death [1].

Scanning electron microscopy was also used for detecting cells on the surface of aortic walls. The preparations made by the standard method were examined under a scanning electron microscope (JSM-2) fitted with 200-μ aperture at 25 kV and the sample slope of 0-40°.

Adhesion of rat cells to devitalized aortic walls and aortic valve leaflets was evaluated by the loss of cells in the culture medium after inoculation of cell suspension on tissue fragments and by the number of live cells adhered to tissue, which were detected by fluorescent microscopy. After inoculation the cells were cultured for 2 days. Inoculation density was 5×10^4 cell/cm^2, which corresponded to the density of monolayer of flattened cells. The culture medium was replaced with fresh portion 24 h after inoculation.

Calcification of the transplants *in vivo* was studied by subcutaneous implantation to rats [2,3,9]. Fragments of aortic walls (5×5 mm^2) were placed into stainless steel porous boxes (50 μ pores) and implanted to Wistar rats under the skin on the back. Before implantation the rats were narcotized with thiopental (3 mg/100 g).

Control and experimental groups consisted of young males weighing 180±10 g kept under standard vivarium conditions with free access to water and food. Vitamin D_3 was added to their rations. The aim of the study and experimental protocol were approved by the Ethics Committee of Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences.

The content of mineralized calcium in tissue fragments after implantation was measured by atomic absorption spectroscopy. After explantation the tissues were washed for 24 h in Ca^{2+}-free saline, dried for 2 h at 100°C, dry weight of the samples was measured, mineralized Ca was dissolved in 0.1 n HCl, and mineralized Ca was measured using an AAS-5100/Zeeman device (Perkin-Elmer). The results were averaged by 10-12 specimens implanted to different rats, the mean square deviation was determined. The significance of differences between the means was evaluated using Student's test. The experiments were performed in at least 3 repetitions.

RESULTS

According to the results of fluorescent microscopy, about 90% fibroblasts in porcine aortic valve leaflets and about 70% SMC in aortic walls were viable after collection. A monolayer of endothelial cells, mainly live, was observed on the surface of the valvular leaflets and aortic intima. We previously showed [2] that enzyme treatment of the aortic walls and leaflets by a previously described method [15] destroyed donor cells. Incubation of aortic wall and leaflet fragments with 0.01% digitonin in DMEM led to the death of all cells within 1-2 h, though cell nuclei remained intact. Digitonin added into the incubation solution with enzymes accelerated cell death and destruction.

The cells adhered onto devitalized fragments of the leaflets and aortic walls within 10-15 min after inoculation. The duration of cell adhesion to the surface of culture plates was also no longer than 10 min. Fluorescent analysis showed that 2 days after inoculation the cells obtained from the rat aortic walls and bone marrow remained on the surface of the leaflets and aortic walls. About 2% cells were at the stage of metaphase and anaphase, similarly as cultured cells inoculated on culture dishes (Fig. 1).

We previously found that native porcine or human aortic walls were greatly calcified after subcutaneous implantation, in contrast to valvular leaflets which did not calcify [1]. For example, the content of mineralized calcium in native porcine aortic walls and heart valve leaflets before implantation was 0.8±0.6 and 0.6±0.6 mg/g dry tissue, respectively, while 2 months after implantation the corresponding value were 69±10 and 0.6±0.6 mg/g dry tissue. This is in line with clinical data indi-

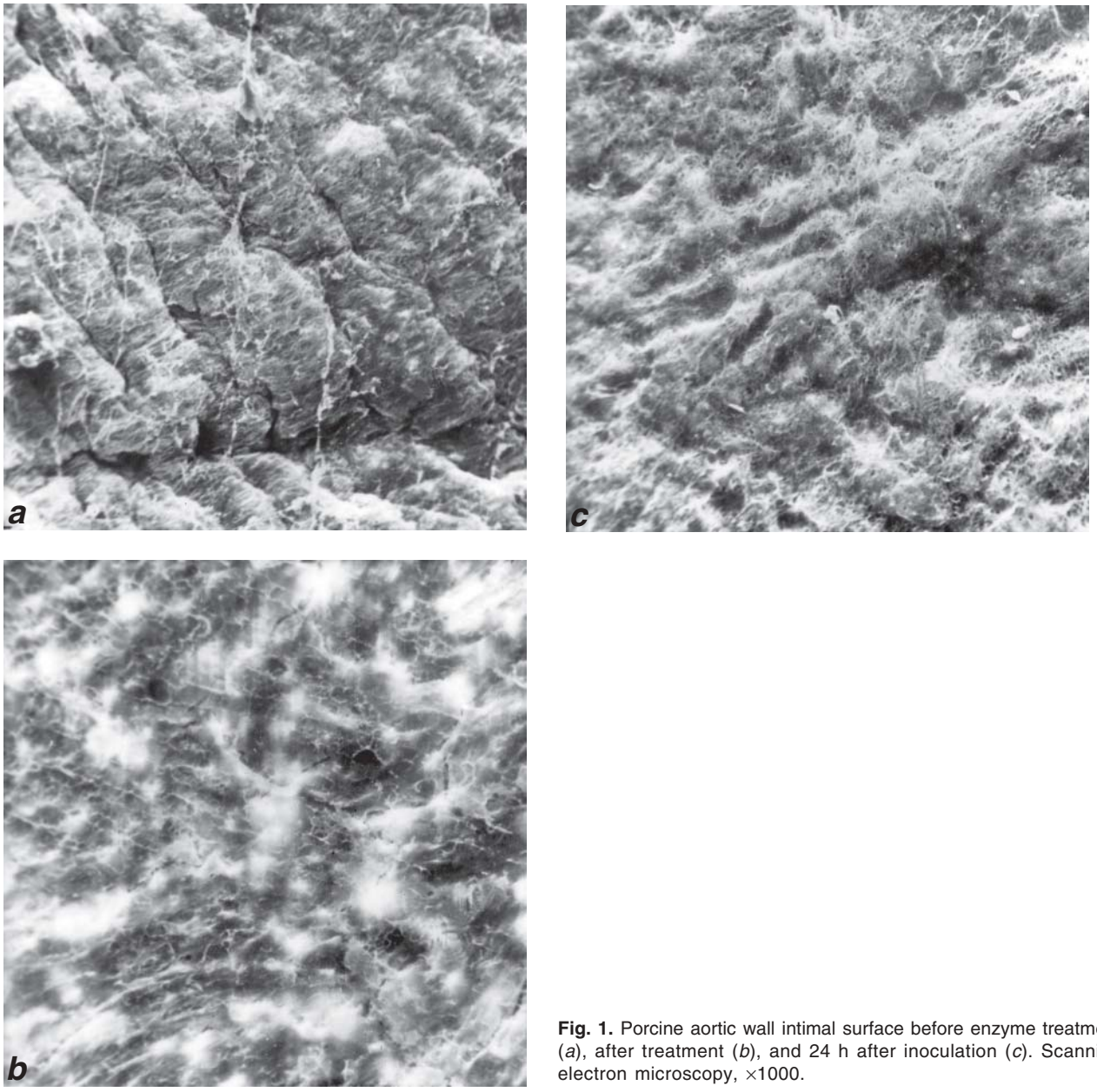

Fig. 1. Porcine aortic wall intimal surface before enzyme treatment (*a*), after treatment (*b*), and 24 h after inoculation (*c*). Scanning electron microscopy, ×1000.

cating significant calcification of aortic walls and with the absence of calcification of the leaflets in orthotopic transplantation of human heart valve allografts [16], which prompts using the model of subcutaneous implantation to rats for studies of calcinosis of allo- and xenotransplants.

The intensity of aortic wall calcinosis observed after devitalization by the method used in this study virtually did not differ from that in native implanted tissues (83±10 mg Ca/g dry tissue; Fig. 2). On the other hand, we observed no calcification after subcutaneous implantation to Wistar rats either in the native, or in devitalized, or in devitalized leaflets with subsequent repopulation with cells from Wistar rat aorta or bone marrow. In all these cases the content of mineralized calcium 2 months after subcutaneous implantation did not surpass the basal level (about 0.60±0.55 mg/g dry weight). Inoculation of cells from the aortic wall of Wistar rats on porcine aortic wall fragments devitalized by three different methods significantly (2-4-fold) reduced their calcinosis after subcutaneous implantation to rats (Fig. 2). The aim of our study did not include saturation of the transplant with cells and migration of inoculated cells inside the tissue matrix. According to our estimations, the count of inoculated live cells before implantation was about 50-fold lower than in native tissue. However, calcinosis significantly decreased even with this little number of cells. The effect could be due to secretion of matrix

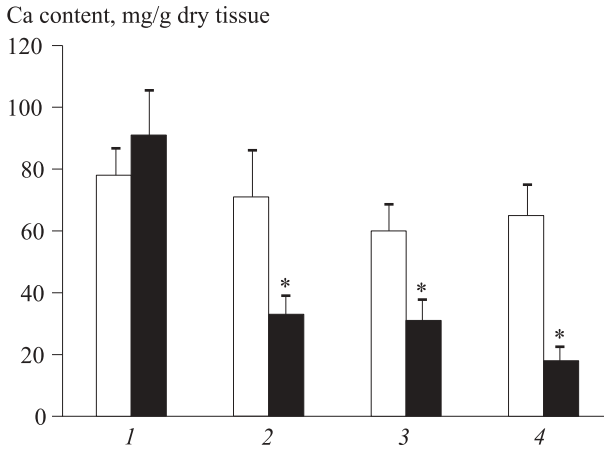

Fig. 2. Effect of cell inoculation on calcinosis of porcine aortic wall fragment subcutaneously implanted to Wistar rats for 2 months. *1*) fragments devitalized by enzyme treatment with trypsin and nucleases with subsequent inoculation of Wistar rat bone marrow cells or without inoculation; *2*) fragments devitalized by enzyme treatment with trypsin and nucleases with subsequent inoculation of Wistar rat aortic cells or without inoculation; *3*) fragments devitalized by digitonin with subsequent inoculation of Wistar rat aortic cells or without inoculation; *4*) fragments devitalized by enzymatic treatment with trypsin and nucleases+digitonin with subsequent inoculation of Wistar rat aortic cells or without inoculation. Light bars: without inoculation of Wistar rat aortic cells; dark bars: cell inoculation. *$p<0.05$ compared to the control.

components by cells, for example glycosaminoglycans, which could prevent the appearance of mineralization centers in tissue matrix [3]. Local oxidation of the culture medium by cells could also prevent calcinosis. Our data suggest that increasing the number of inoculated cells can improve the efficiency of suppression of calcinosis in heart valve and vascular transplants after destruction of donor cells and inoculation of cells from recipient vessels. This necessitates improvement of methods for cell inoculation and culturing, providing their overall migration into the transplant tissue matrix.

Inoculation of cells from Wistar rat bone marrow to devitalized fragments of porcine aortic wall did not reduce their calcinosis after subcutaneous implantation to rats (Fig. 2). Hence, inoculation of a certain cell type is essential for reducing calcinosis, for example, vascular cells. Presumably, the collected population of bone marrow cells contained osteogenic precursor cells, which could promote calcinosis. It is also possible that calcinosis-suppressing cell population can be isolated from the bone marrow, but further studies are needed to solve this problem and to clear out the possibility of using other cell types, for example, fibroblasts, fatty tissue stromal cells, *etc.*

We inoculated isogenic aortic cells on a xenotransplant as a model of *in vitro* repopulation of the transplant by recipient cells, which promoted reduction of calcinosis in the implanted tissue. The results indicate that reduction of calcinosis can be expected after inoculation of autologous vascular cells on the transplant.

Hence, our findings indicate good prospects of inoculation of cells from recipient vessels on devitalized xeno- and allotransplants of heart valves and vessels with the aim of reducing their calcinosis and improvement of biocompatibility.

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