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Morphological Evaluation of the Tissue Reaction to Subcutaneous Implantation of Decellularized Matrices

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Based on the data of morphological analysis, we performed histological evaluation of rat tissue reaction to subcutaneous implantation of decellularized matrices of intrathoracic organs and tissues. Cell composition of the inflammatory infiltrate was analyzed, and the dynamics of macrophage and T and B lymphocyte content was assessed on days 7 and 14 of the experiment. It was found that the reaction to implantation depended not only on the quality of decellularization and efficiency of removal of antigen molecules, but also on the original histological structure and quality of preimplantation processing of the transplant.

Key words: *decellularization; biological scaffolds; tissue engineering; rats*

At present, various techniques and methods of fabrication of decellularized matrices based on physical, chemical, and enzymatic treatment are proposed. The resultant structure does not contain cells and products of their degradation, but retain intricate geometry of the organ, including relatively intact vasculature [6,8].

The method of obtaining decellularized matrix largely determines the tissue reaction of the recipient to implantation of the tissue engineering construct. It is of crucial importance to find a balance between complete elimination of antigen molecules and retaining of the qualitative composition of extracellular matrix (ECM) proteins of the organ [5].

An important step in studying the quality of decellularization of organs is subcutaneous implantation of the scaffolds. This procedure helps to determine *in vivo* the efficiency of elimination of cell-associated proteins and to evaluate their contribution to the tissue response to implantation. Moreover, local tissue response to aggressive chemical substances used during decellularization and probably persisting in the scaffold in complexes with ECM proteins [4,9].

Here we compared biocompatibility, biodegradation, local tissue reaction to implantation of decellularized extracellular matrices of intrathoracic organs.

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MATERIALS AND METHODS

The study was performed on 20 male Wistar rats weighing 210±40 g at the Laboratory of Fundamental Research in Regenerative Medicine, Kuban State Medical University, in accordance with the Regulations for Animals Experiments (Order No. 755 of the Ministry of Health of the USSR, August 12, 1972) and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986); the study protocol was approved by Local Ethical Committee (protocol No. 21/1).

Decellularization of the diaphragm, lungs, and heart was performed by modified protocols using sodium deoxycholate and DNase [1,3,7]. In our previous studies, acellular matrices containing no intact cells and cell nuclei and consisting of ECM proteins types I and IV collagens, laminin, elastin, and fibronectin were obtained. Before transplantation, the fragments of these matrices (0.5×1 cm) were sterilized for 15 min in 10% ethanol and washed 3 times in sterile PBS.

The animals were anesthetized with Zoletil 100 (6 mg/kg) and xylazine (0.5 ml/kg, Rometar, Spofa) and subcutaneous implantation of decellularized ma-

trices of the lung (group 1), diaphragm (group 2), and heart (group 3) was performed in the interscapular region. The rats were sacrificed with barbiturate overdose (150 mg/kg) on days 7 and 14 after the implantation and the organ complex was isolated.

The implants with surrounding tissue were surgically isolated, fixed in 10% neutral formalin, and dehydrated routinely using a Leica TP1020 tissue processor, and then embedded in paraffin according to a standard procedure using a EG1150H station. For histological analysis of the preparations, 4-μ paraffin sections were sliced on a RM2235 rotation microtome (Leica), mounted on highly adhesive glasses, dewaxed, rehydrated, and stained with hematoxylin and eosin (Sigma-Aldrich). Qualitative immunohistochemical analysis of the cell infiltrate around the implant was performed. To this end, the sections were deparaffinized and rehydrated, endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, and antigens were retrieved in citrate buffer (ab64236, Abcam) in a water bath for 40 min. The following primary antibodies were used: anti-CD3 (ab16669), anti-CD8 (ab33786), and anti-CD20 (ab85809); all antibodies were purchased from Abcam. The preparations were poststained with Mayer's hematoxylin for 10 sec. Rabbit specific HRP/DAB (ABC) Detection IHC Kit (ab 64261, Abcam) was used. For the morphological detection of activated proinflammatory macrophages, immunohistochemical reaction with antibodies to mannose receptor of macrophages (ab64693, Abcam) was performed. In this case, we used a fluorescent detection system with secondary Goat polyclonal Secondary Antibody to Rabbit IgG-H&L (Alexa Fluor 488, ab150081, Abcam; 1:500).

The preparations were examined under an Olympus CX 41 microscope. The cells demonstrating positive reaction with antibodies to the studied receptors were counted manually on paraffin sections in 4-5 fields of view at ×400.

RESULTS

In group 1 (implantation of acellular matrix of the lung), a thin (up to 500 μ) connective tissue capsule around the implant was formed by day 7; it contained plethoric blood vessels, proliferating fibroblasts, and mononuclear cells. Pores of the material were filled with fibrin and infiltrated with lymphocytes and macrophages with minor admixture of neutrophils. In the adjacent tissues, edema, moderate inflammatory infiltration, proliferating fibroblasts, and newly formed vessels were seen. On day 14, significant decrease in the implant in size was observed due to reduction of edema, compaction and more dense arrangement of collagen fibers in the acellular matrix. Newly formed thin-walled vessels grew into the implant. No ECM degradation was observed. The degree of inflammatory reaction significantly decreased both in the implant and adjacent tissues. At the early terms, the infiltrate contained different cells, including T and B lymphocytes ($CD3^+$ and $CD20^+$ cells, respectively) and proinflammatory fraction of macrophages (positive reaction with antibodies to mannose receptor), but on day 14, we observed only a small amount of B cells (Table 1; Fig. 1).

In group 2 (implantation of acellular matrix of the diaphragm), the matrix in 7 days retained the porous structure. A granulation tissue capsule (up to 900 μ thick) was formed around the matrix and fibroblast fibers grew into the implant. Intensive inflammatory reaction seen, the infiltrate contained mononuclear cells (T and B lymphocytes and macrophages) and segmented leukocytes, including eosinophils. In tissues surrounding the capsule, pronounced edema and plethoric blood vessels, especially around the implant, were seen. On day 14, edema became less pronounced, ECM of the diaphragm underwent compaction, degradation, and resorption by macrophages; the growth of newly formed vessels and productive capillaritis were observed. The inflammatory reaction both in the sample and in surrounding tissues was considerably less pronounced due to a decrease in the number of activated macrophages and T cells. Similar to implantation of decellularized lungs, the content of B cells in the infiltrate did not appreciably change (Table 1; Fig. 2).

Morphological analysis of group 3 samples (implantation of decellularized matrix of the heart) re-

TABLE 1. Morphological Analysis of the Cell Infiltrate Surrounding the Implants

Implant	Anti-mannose receptor, %		CD3, %		CD8, %,		CD20, %	
	day 7	day 14	day 7	day 14	day 7	day 14	day 7	day 14
Decellularized diaphragm	27.4	4.7	37.5	13.9	32.1	5.4	44.8	38.8
Decellularized lung	26.2	2.1	34.4	12.1	13.6	0.5	32.4	30.7
Decellularized heart	23.6	13.7	39.1	24.4	16.4	19.7	36.6	48.7

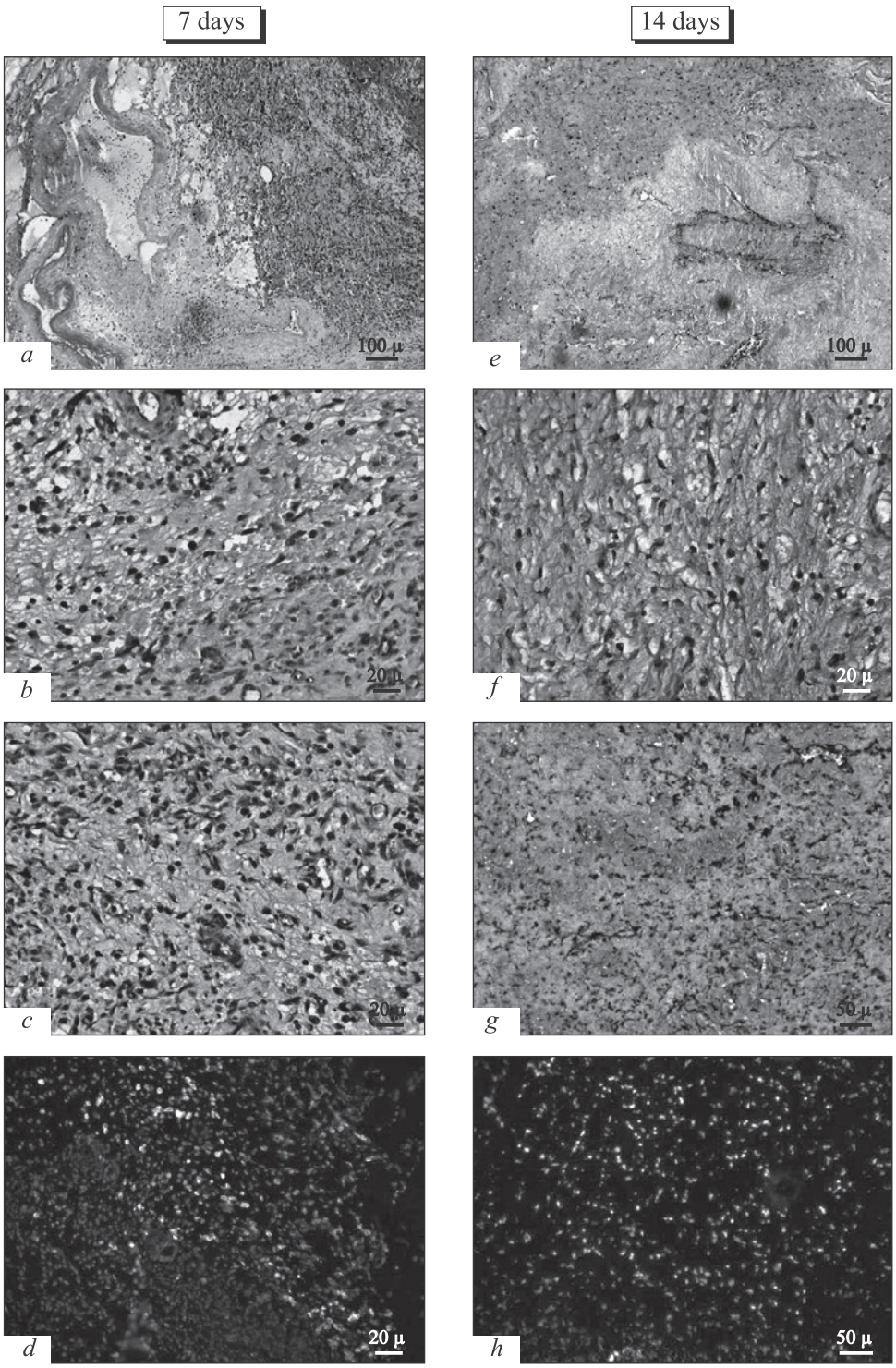

Fig. 1. Decellularized matrix of the lung on days 7 and 14 after transplantation. Hematoxylin and eosin staining (*a*, *e*), immunohistochemical reaction with antibodies to CD3 T lymphocyte receptor (*b*, *f*), CD20 receptor of B lymphocytes (*c*, *g*), and mannose receptor of macrophages (*d*, *h*); ×100 (*a*, *e*), ×200 (*d*, *h*), ×400 (*b*, *c*, *f*, *g*).

vealed the development of pronounced inflammatory reaction at the site of surgery. On day 7, the implant was completely surrounded by demarcation inflammation up to 2 mm thick consisting of segmented leukocytes, B and T lymphocytes, and proinflammatory activated macrophages. Neither fibroblast proliferation, nor connective tissue capsule formation around the implant was seen. By 14 days, the implantation zone was also abundantly vascularized (up to 5-6 vessels in the field of view at ×400) and surrounded by dense

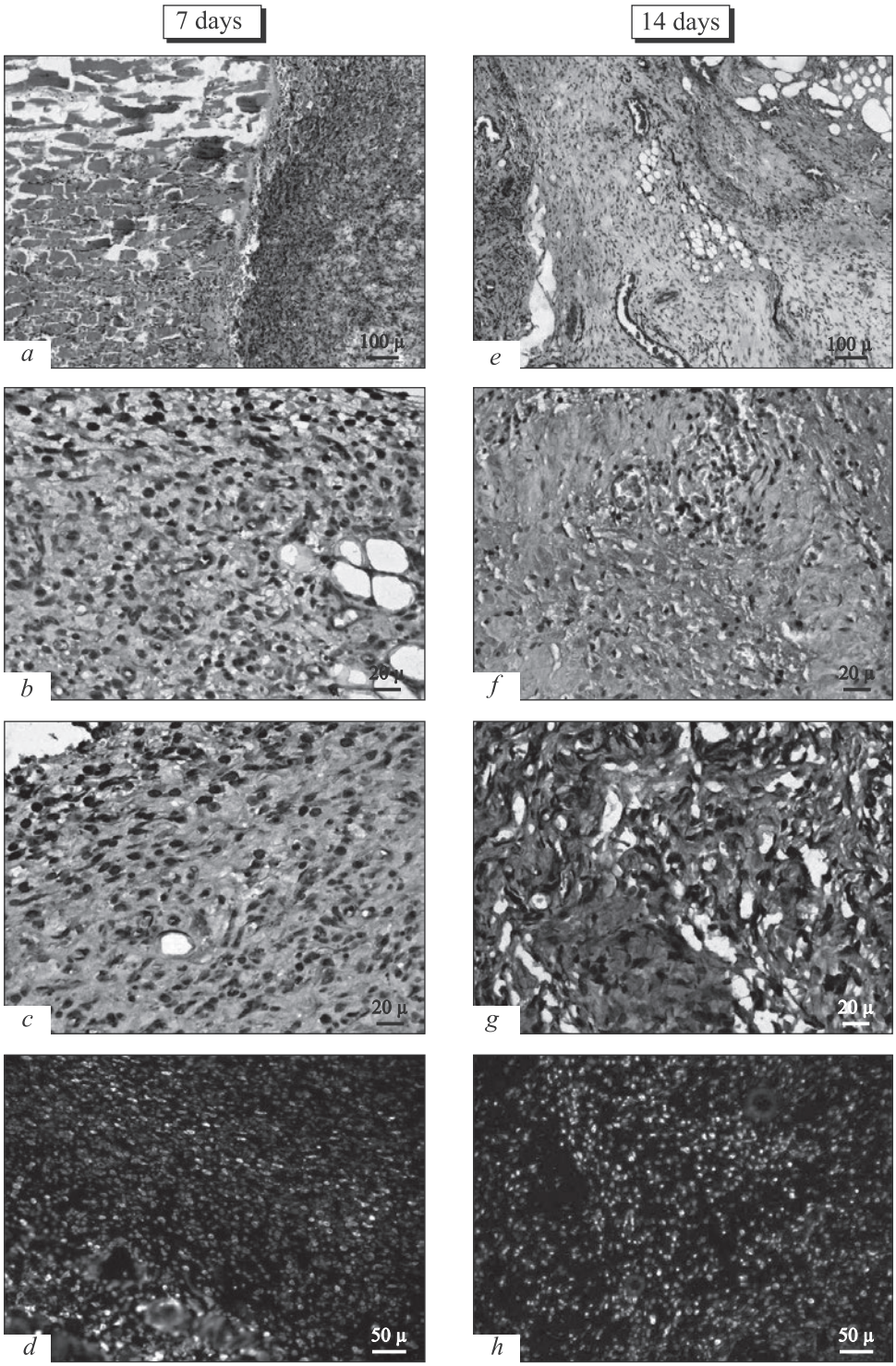

Fig. 2. Decellularized matrix of the diaphragm on days 7 and 14 after implantation. Hematoxylin and eosin staining (*a*, *e*), immunohistochemical reaction with antibodies to CD3 T lymphocyte receptor (*b*, *f*), CD20 receptor of B lymphocytes (*c*, *g*), and mannose receptor of macrophages (*d*, *h*); ×100 (*a*, *e*), ×200 (*d*, *h*), ×400 (*b*, *c*, *f*, *g*).

fibrous capsule with a low number of vessels. Against to the background of reduced number of T and activated macrophages (by 38 and 42%, respectively), the content of B lymphocytes in the infiltration inside and around the implant increased by ~30% (Table 1; Fig. 3).

These results suggest that subcutaneous implantation of decellularized matrices can be considered as an important step of *in vivo* assessment of tissue response of the recipient organism to implantation of the tissue engineering constructs. The questions remain unan-

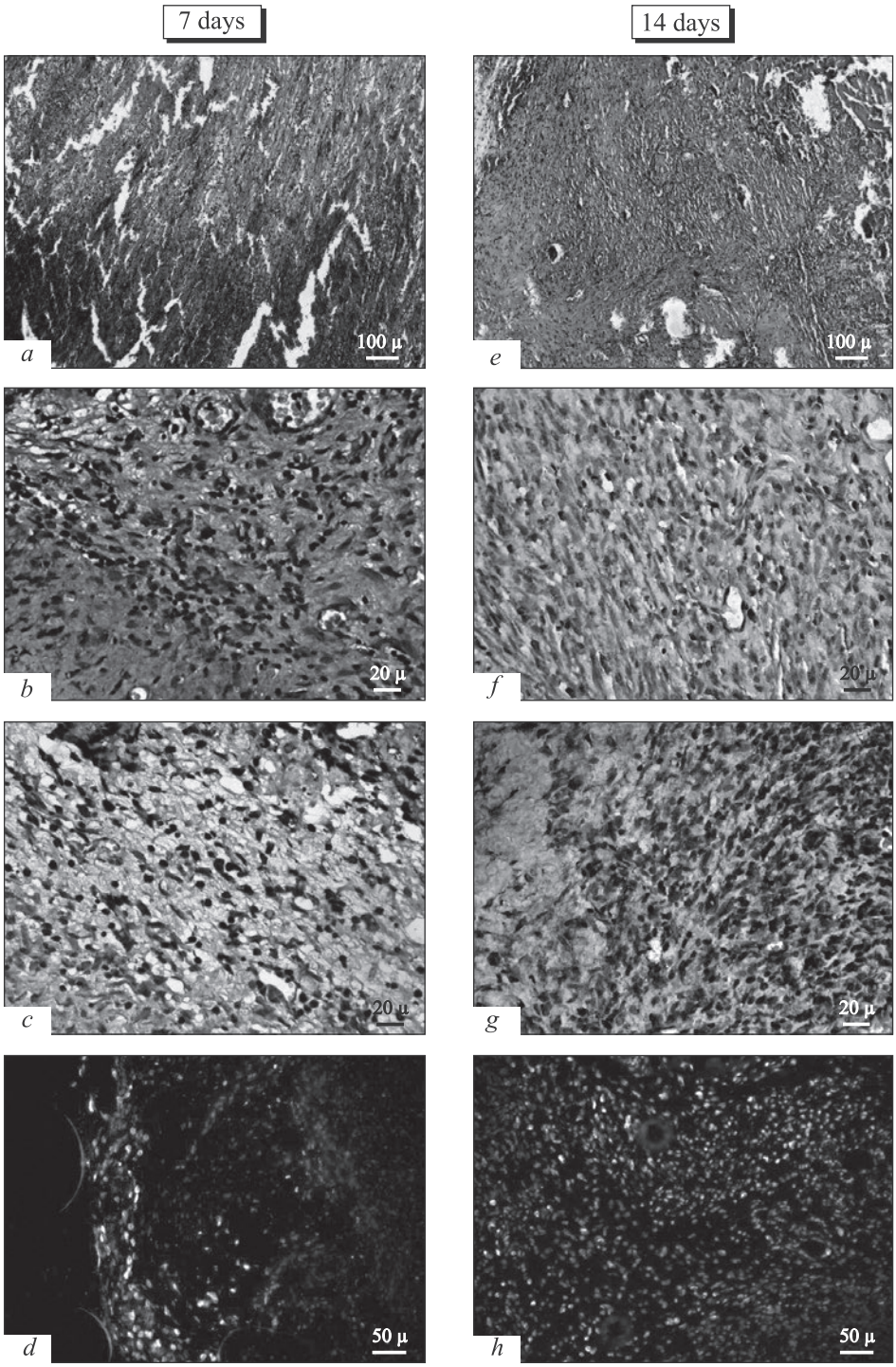

Fig. 3. Decellularized matrix of the heart on days 7 and 14 after implantation. Hematoxylin and eosin staining (*a*, *e*), immunohistochemical reaction with antibodies to CD3 T lymphocyte receptor (*b*, *f*), CD20 receptor of B lymphocytes (*c*, *g*), and mannose receptor of macrophages (*d*, *h*); ×100 (*a*, *e*), ×200 (*d*, *h*), ×400 (*b*, *c*, *f*, *g*).

swered whether the tissue source or the method of processing is main factor determining the reaction of the recipient [3].

For our study, we have intentionally chose matrices of organs with different histological structure (lung, diaphragm, and heart) processed by similar techniques. Our previous studies aimed at *ex vivo* evaluation of these ECM showed that they fully met modern criteria of decellularization [2].

Our experiments demonstrated different tissue response of the recipient to ECM implantation. The lung ECM induced least expressed response, success-

fully integrated into the tissues, and underwent no significant changes. In group 1, the number of macrophages and T lymphocytes tended to decrease, which indicates good biocompatibility and low immunogenicity and toxicity of the matrices. Implantation of ECM of muscular organs, *e.g*. diaphragm and heart, was associated with more pronounced macrophage resorption of the matrix and induced more intense inflammatory response. In groups 2 and 3, the content of segmented leukocytes was significantly higher than in group 1. However, in our previous studies with orthotopic transplantation of the diaphragm we did not observe this morphological pattern [7], which raises new questions about differences in the response to hetero- and orthotopic transplantation of the implant. There was also a tendency to an increase in the content of B lymphocytes, appearance of eosinophils in the infiltrate, against the background of decreased content of macrophages and T lymphocytes. We attribute these differences to the more dense structure and, as a consequence, incomplete washing of the matrix from detergents and enzymes, and favorable conditions for bacterial contamination. Thus, the tissue response to implantation depends not only on the quality of decellularization and efficiency of removal of antigen molecules, but also on the quality of the preimplantation processing of the sample.

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