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ORIGINAL ARTICLE

Transplantation of Poly-L-Lactide Film with Adhered Fibroblasts Accelerates Tissue Regeneration of Ischemic Skin Defects

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ABSTRACT
Tissue-engineered skin with mechanical and biological properties that correspond to native tissue is becoming a valuable tool for the treatment of non-healing chronic skin defects in blood flow disturbances. The study was performed on 14 mice of the C57/B1 line aged between 5 and 7 months. The animals were divided into the control and the experimental group with 7 mice in each one. A model ischemic wound on the skin of experimental animals was covered with a poly (L-lactide) film with attached allogenic fibroblasts and then with an aseptic VoscoPran dressings with levomekol. On day 12, a scar was intraoperatively excised, embedded in paraffin and stained with H&E by the Weigert-Van Gieson method. A degradable polylactide film with allogenic fibroblasts cultured on it accelerates the wound healing process by 31.41%, taking into account the time of epithelialization of the wound, eruption of the ring-retaining seams, and the falling off of the ring itself (on day 8.5±0.1 days versus on day 12.4 ± 0.10 in the controls). At that, the thickness of the epidermis against the background of wound closure with a polylactide film with allofibroblasts was on average less by 2.63% than in the control group but it was more differentiated and gave rise to skin derivatives, hair. The granulation tissue of the regenerating skin defect in the experimental group showed the initial signs of fibrosis without inflammatory cell infiltration. Collagen fibers acquired an ordered arrangement, their area increased by 16.2%, and vascularization decreased considerably (by 30.43%). On day 12 of wound healing, the thinned remains of the film could be found only in the center of the wound above the formed epidermis and the scab was completely absent. Thus, the biodegradable polylactide film with allogenic fibroblasts cultured on it improves significantly the microscopic parameters of the wound healing process and the macroscopic signs of healing of an ischemic skin defect.
Keywords*: cutaneous wound, polylactide film, fibroblast, regeneration, angiogenesis.*

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INTRODUCTION

Despite the better understanding of the morphological picture of skin defect healing in blood flow disturbances, persistent non-healing defects remain a serious public health problem [1]. Tissue engineering is considered an alternative approach to treating such skin wounds [2]. Tissue-engineered skin with mechanical and biological properties that correspond to native tissue is becoming a valuable tool for the treatment of non-healing chronic wounds [3]. Fibroblasts grown on a suitable biodegradable scaffold are a possible strategy for creating a dermal substitute, due to which epithelization can occur naturally [1, 4]. Currently, a wide range of different biodegradable scaffolds has been suggested and tested: Sacran Hydrogels [5], fibrin gel [6], fibrin-coated electrospun polylactide nanofibers [7], electrospun polycaprolactone collagen nanofibrous membrane [8]. It has become clear that superficial and mechanical properties of such biomaterials determine proliferative, migratory, secretory, and other activities of the cells cultivated on them [9, 10]. Aliphatic polyesters, also known as poly (hydroxyesters), are one of these biologically absorbable and biocompatible groups of polymers that have great potential for use in skin tissue regeneration [11]. This class of polymers includes poly (lactic acid) (PLA). Of these,

the most widely used polylactides are poly (L-lactide) (PLLA) and poly (D-lactide) (PDLA), respectively [12]. However, at present, the attention of researchers is focused on the study of cell reproduction and growth on polymer matrices with different spatial organization and after applying the components of the intercellular matrix in order to ensure and improve the formation of the structural basis of differentiating tissues [13, 14]. Works on morphological transformations in tissues of non-healing skin defects in case of blood flow disturbances after transplantation of a polylactide film with allogenic fibroblasts are rare and do not analyze the problem at different stages of the wound healing process.

To study the morphological structure, collagen formation and angiogenesis in the biopsy specimens of the newly formed epidermis and dermis on the 12th day after closure of the model ischemic skin defect with polylactide film associated with dermal fibroblasts.

MATERIAL AND METHODS

Experiment Design

The study was performed on 14 mature white mice of the C57/B1 line aged between 5 and 7 months kept in the vivarium of Medical Academy named after S.I. Georgievsky. The animals were divided into the control and the experimental group with 7 mice in each one. The experiments were carried out taking into account all the principles of humanity included into Directive 2010/63/EU and in accordance with the guidelines of ICMR on animal research (2006). An ischemic cutaneous wound was made in the scapular region of the mice of both groups through operative procedure. In the experimental group, dermal fibroblasts were obtained using fermentation process and cultured in a DMEM/F_{12} medium (Lonza) [15].

Cover glasses size 15 mm x 15 mm with a hydrophilic (untreated) surface were used as a substrate to apply a solution of poly-L-lactide (Poly-(L-lactide) from Aldrich, USA). The uniform distribution of polylactide on the glasses was obtained due to the use of a polar and water-retaining solvent, acetone. 30 µl of the polymer solution was applied so that its concentration on the glass was 1.4µg / mm^2. The concentration of the polymer solution of 50 mg/ml with 85 µl applied to the surface of the cover glass was considered optimal. The film thickness was 100 µm [16]. The prepared cover glasses with polylactide were dried and sterilized using ultraviolet light for 2 hours.

Allogenic fibroblasts of mice (Passage 2) were seeded on cover glasses coated with polylactide in an amount of 3x103 cells/cm^2 in a DMEM/F_{12} medium (Lonza) with the addition of 10% Fetal Bovine Serum (HyClone). They were cultured in an incubator in an atmosphere containing 5% CO_2 at a temperature of 37°C. Changes in the shape of the cells during their cultivation on the polymer were examined under an inverted OLIMPUS CX-41 microscope.

After reaching confluent, the polylactide film was separated from the cover slip and transplanted intraoperatively into a model skin defect in the interscapular region of the mice of the experimental group (Figure 1). The surface of the film with attached allogenic fibroblasts was applied to the wound surface. The ends of the film were sown to a silicone ring. Then the wounds in both control and experimental groups were covered with aseptic VoscoPran dressings with levomekol.

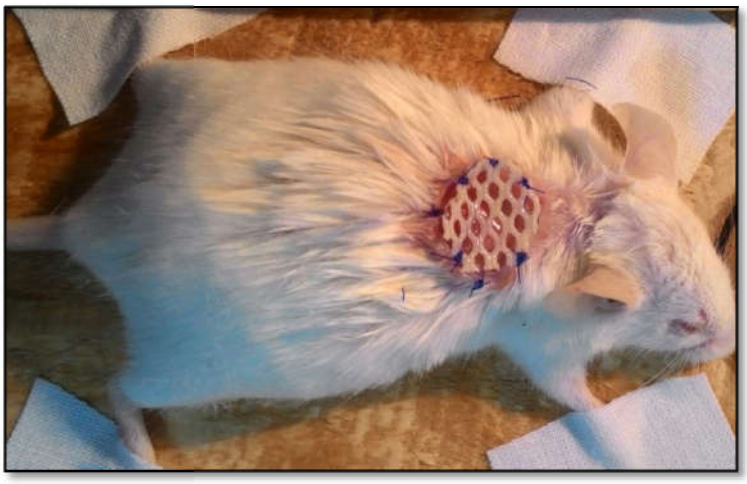


Figure 1. A model ischemic wound on the back of the mouse. The edges of the wound are sewn to the silicone ring. The wound is covered with a poly (L-lactide) film with attached xenogenic fibroblasts and then with an aseptic VoscoPran dressing with levomekol.

The Morphological Study of Scars

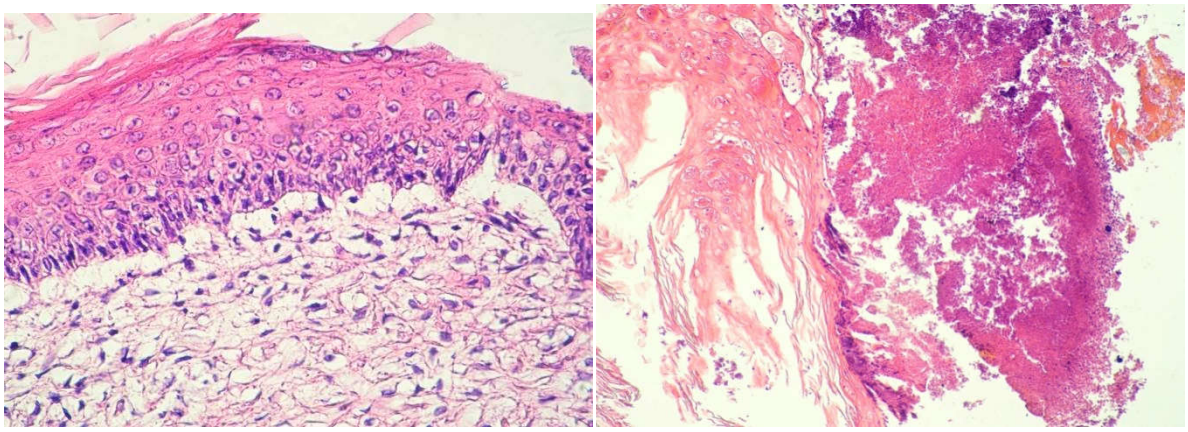
On the 12th day after the operation, in all groups, the formed scar was intraoperatively excised and fixed in a 10% buffered formalin solution for morphological examination. The material was embedded in paraffin and stained with H&E and by the Weigert-Van Gieson method to visualize the elastic and collagen fibers. Morphological examination of histological preparations was carried out with a OLIMPUS SX-31 light-optical microscope. The thickness of the epidermis, the number of microvessels in the sections, the area of collagen fibers and microvessels in the dermis of the scars were determined using an image analysis program (ImageJ 1.46r, National Institutes of Health, USA). We use a 10X ocular lens and a 40X objective lens (a total magnification of 400X). Fifty measurements were performed in each group. The obtained digital data (expressed in pixels) were *converted* into μm by using special coefficients: 6379251 for a 10X lens and 98911797 for a 40X lens.

Statistical Analysis

We used the statistical software «Statistica». (v6.0, StatSoft, USA) and Microsoft Excel 2007 to carry out statistical analysis. Baseline characteristics were summarized as frequencies and percentages for categorical variables and as mean±SEM for continuous variables. The Mann-Whitney (U Test) was used to compare the differences between the two independent groups. A probability value of P=0.05 was considered statistically significant.

RESULTS and DISCUSSION

In the mice of the control group, the silicone ring fell away spontaneously on day 12.4 ± 0.10 after the operation for producing a model wound due to its epithelialization and the eruption of the sutures. The wound was fully epithelialized under the thick remains of the scab. In the sections of the scar, the epidermis appeared as a multilayered incompletely formed epithelium 51.73 ± 0.12 μm thick (Table 1). There was a basal layer and several rows of prickle cells. The stratum corneum was in the initial stages of cell differentiation and was revealed in several areas only (Fig. 2A). The remaining scab consisting mainly of amorphous substances and cellular debris was seen over the epidermis. There were no cellular elements (Fig. 2B). The dermis of the scar did not form the papillae extending into the epidermis and the border between the epidermis and the dermis was vague and even. Edema and a slight leukocyte infiltration were seen under the basement membrane.



A B

Figure 2. The control group. Scars of a mouse skin ischemic wound on day 12 after the surgery for producing a skin defect. A – Section of the scar: 1 - epidermis; 2 - edema under the epidermis; 3 - granulation tissue; 4 - blood capillary. B - Scab over the epidermis: 1 - amorphous substance; 2 - cell debris

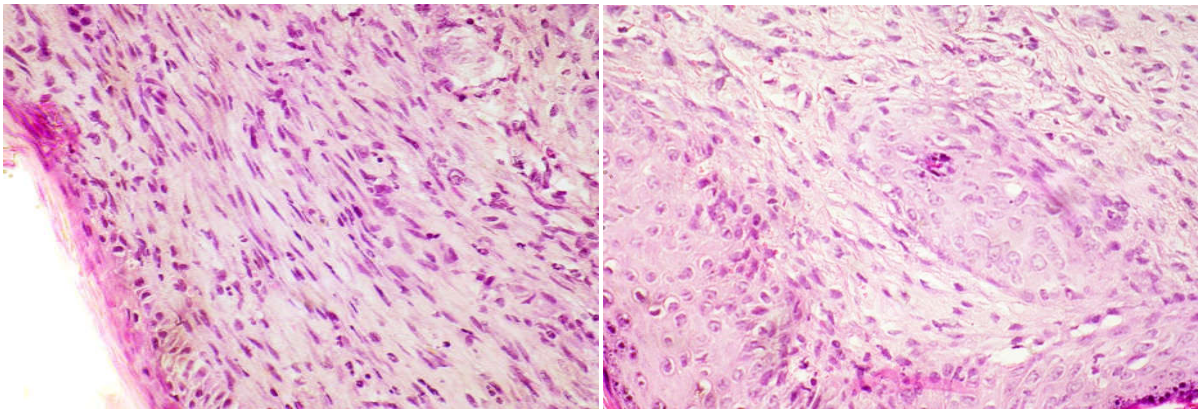
Table 1. Quantitative Characteristics of Scars on Mouse Skin in the Control and Experimental Groups

Skin scars	Epidermis thickness in microns	The dermis area in sections in microns	The area of blood vessels in the dermis in %	The area of collagen fibers in the dermis in %
Skin scars in the control group	51.73±0.12	46259.83±1.20	0.69±0.02	29.70±0.16
Skin scars in the experimental group	50.37±0.14	66030.80±1.05	0.48±0.02	35.44±0.15

The entire skin defect was filled with developing granulation tissue, in which cells of tissue and hematogenous origin were revealed. The papillary and reticular layers of the dermis were not demarcated and consisted of uniformly randomly localized thin collagen fibers forming the reticular structure. There were cells, mainly functionally active fibroblasts, between the collagen fibers. These fibers occupied 29.70 ± 0.16% of the dermis area. There were no elastic fibers. A few blood capillaries and venules were enlarged and their area was equal to 0.69 ± 0.02 μm. Slight leukocyte infiltration could be seen around them.

In mice of the experimental group, the wound became epithelized and the silicone ring fell away on 8.5 ± 0.1 day after the operation and closure of the ischemic skin wound with polylactide film associated with dermal allogenic fibroblasts. This is 31.41% earlier than in control mice.

The whole surface of the wound was covered with epidermis (Fig. 3A). The scab was completely absent. The remnants of the polylactide film were preserved as a thin structureless strip on the surface of the epidermis in the central parts of the wound (Fig. 3B). On day 12 after the transplantation of allogenic fibroblasts on a polylactide film into the wound, the structure of the epidermis at the periphery of the healing wound differed from that in its central sections. The thickness of the epidermis on average was 2.63% less than in the control group. However, the epidermis looked more differentiated compared to the control group, especially along the wound edges. There were three layers: basal, prickle and horny ones. The epidermis formed outgrowths into the underlying granulation tissue, which were hair anlages (see Fig. 3). The epidermis was lower and had fewer rows of cells in the center of the wound. There appeared signs of the papillary dermis in the form of a wave-like border between the basement membrane of the epidermis and the underlying granulation tissue. Granulation tissue was characterized by the presence of initial signs of fibrosis. Oxyphillic bundles of collagen fibers lined up parallel to each other. The area occupied by collagen fibers increased by 16.2% compared with the control group. The shape of the fibroblastic cells changed to fusiform, and they lay between the parallel bundles of collagen fibers. The area occupied by blood vessels decreased by 30.43% compared with the controls, which indicates a decrease in their number and blood supply and the beginning of the process of consolidation of the scar.



A B

Fig 3. Scars of mouse ischemic skin wound on day 12 after surgery and covering with poly (L-lactide) film with attached xenogenic fibroblasts. A - the edge of the scar: 1 - the epidermis; 2 - hair anlage; 3 - fibrosing granulation tissue. B - the central part of the scar: 1 - epidermis; 2 - the remains of a polylactide film; 3 - fibrosing granulation tissue; 4 - collagen fibers. Stained with hematoxylin and eosin. Magnification: x 200.

All the results of morphometric studies are presented in Table 1. Elastic fibers were absent in all parts of the dermis. Leukocyte infiltration was mild and this could be considered as a sign of overcoming the inflammatory phase of the wound healing process.

Our previous work [17] and works of a number of authors [18, 19] showed that the introduction of xenofibroblasts and autofibroblasts into an ischemic model wound also reduced the inflammatory response and healing time. Collagen formation by fibroblasts and vascularization in the granulation tissue improved. However, wound closure with a polylactide film with cultured allofibroblasts is much more effective. This could be explained by the fact that spatial organization and functional properties of the cultured cells might be disturbed when they are transferred to wounds. These problems are associated primarily with the inevitable damage to the prepared cells as a result of their enzymatic processing during the separation of the cell layers from the surface of the vessels in which they are cultivated, which leads to insufficient mechanical strength of cell associations and partial loss of surface receptors [16].

Multiplication and growth of cells on 2D polymer matrices ensure the preservation of the initial state of intercellular contacts and cell surface receptors during the creation of cell products and their transplantation [20], which ensures the faster formation of the structural basis for regenerating tissues.

CONCLUSION

A degradable polylactide film with allogenic fibroblasts cultured on it after its transplantation into a model ischemic skin defect accelerates the wound healing process on day 12 by 31.41%, taking into account the time of epithelialization of the wound, eruption of the ring-retaining seams, and the falling off of the ring itself (on day 8.5±0.1 days versus on day 12.4 ± 0.10 in the controls). At that, the thickness of the epidermis against the background of wound closure with a polylactide film with xenofibroblasts was on average less by 2.63% than in the control group but it was more differentiated and gave rise to skin derivatives, hair. The granulation tissue of the regenerating skin wound in the experimental group showed the initial signs of fibrosis without inflammatory cell infiltration. Collagen fibers acquired an ordered arrangement, their area increased by 16.2%, and vascularization decreased considerably (by 30.43%). On day 12 of wound healing, the thinned remains of the film could be found only in the center of the wound above the formed epidermis and the scab was completely absent. Thus, the biodegradable polylactide film with allogenic fibroblasts cultured on it improves significantly the microscopic picture of the wound healing process and the macroscopic signs of healing of an ischemic skin defect.

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