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ORIGINAL ARTICLE

Allogenic fibroblasts in collagen-based dermal equivalent change ultramicroscopic characteristics of the cells of granulation tissue by the 12th day of the healing of cutaneous wound against the background of hemodynamic insufficiency

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ABSTRACT
Transplantation of large constructs of allogenic fibroblasts and natural components of their microenvironment is currently used as replacement therapy to stimulate the regeneration potential of skin defects complicated by hemodynamic insufficiency. Ultramicroscopic characteristics of the main cells in the regeneration process has been described poorly. The aim of the research was to study the ultrastructural features of cells on day 12 after transplantation of the dermal equivalent with allogenic fibroblasts into the ischemic cutaneous wound. The study was performed on 14 mice of the C57/B1 line aged between 4 and 6 months. The dermal equivalent with allogenic fibroblasts was transplanted into a surgical model wound in the scapular region. Ultrathin sections were made on the UMPP-7 Ultratome (Ukraine) and examined under a Selmi electron microscope (Ukraine) at an accelerating voltage of 125 kV. Morphological examination of semi-thin sections was carried out using an OLYMPUS CX31 light-optical microscope with an OLYMPUS C-5050Z digital camera. On day 12 day of wound healing, significant differences in the amount and size of the cells, as well as in the area they occupied in the granulation tissue of the peripheral and central regions were found. After transplantation of the dermal equivalent with allogenic fibroblasts, the number of cells increased, the average cell area and the average area of all cells decreased. Signs of edema and neutrophils were absent; there was an active degradation of macrophages, indicating a low level of inflammatory response. In the center of the healing wound, binucleated myofibroblasts were found. At the periphery of the wound, granulation tissue was in the initial stage of remodeling, with myofibroblasts prevailing, which provided contraction of the wound. The dermal equivalent accelerates healing of skin defects complicated by hemodynamic deficiency.
Keywords: *skin defect, regeneration, granulation tissue, tissue engineering.*

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INTRODUCTION

Repair of ischemic persistent non-healing skin defects is still an important medical problem [1]. To solve it, many different medical, physiotherapeutic and surgical methods have been suggested and are used with varying degrees of success, both in isolation and in various combinations [2]. To date, a fundamentally new way to optimise wound healing process has been suggested based on a currently developed method of cell replacement therapy [3]. At that, transplantation of additional cells of mesenchymal origin into the damaged area is a method of choice and does not contradict the theoretical provisions on the evolutionarily fixed tissue determination [3]. The first phase of wound healing process, the inflammatory phase, is usually delayed in a chronic wound, which causes signs of all the three phases

of the wound healing to be present simultaneously [4]. A search for ways to reduce the duration of inflammation has revealed that human dermal fibroblasts are a source of adiponectin, which acts as an active anti-inflammatory cytokine and induces the production of anti-inflammatory factors, such as IL-10 and IL-1RA [5, 6]. The dermis of the skin contains the so-called ordinary loose connective tissue, where type-I collagen is the main protein component and fibroblasts are the main cell pool, mainly responsible for its biosynthesis and remodeling. Creation of an artificial construct, the dermal equivalent, on basis of these major components was an important step in treatment of non-healing ischemic wounds, which closes the skin defect and creates optimal conditions for the proliferation and functioning of the main cellular elements in the skin regeneration process [7]. However, the findings of the ultrastructural features of cellular elements in transplantation of the dermal equivalent are sporadic and scarce, which makes our research relevant.

The aim of the research was to study ultrastructural characteristics of cells on day 12 after transplantation of the dermal equivalent with allogenic fibroblasts into the ischemic cutaneous wound.

MATERIAL AND METHODS

The study involved 14 mice of the C57/B1 line aged between 4 and 6 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 animals 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 [8]. In the experimental group, dermal fibroblasts were obtained using fermentation process and cultured in a DMEM/F12 medium (Lonza). The second passage cells were used to form the dermal equivalent. The dermal equivalent was prepared on basis of Type I collagen extracted from rat tails. Sterile 0.34 M NaOH solution was combined with a concentrated (×10) 199 nutrient medium in a ratio of 1: 1. The resulting mixture was supplemented with a cooled collagen solution, after which a suspension of fibroblasts was added into the DMEM/F_{12} medium containing 10% fetal serum (HyClone). The resulting mixture was incubated at 37° C in an incubator until the gel was completely polymerized [9]. The finished tissue-engineered construct was transplanted into the ischemic cutaneous wound in the mice of the experimental group [9].

On the 12th day after the operation, the resulting scars in the mice of both groups were intraoperatively excised and fixed with glutaraldehyde in phosphate buffer. Samples for ultramicroscopic examination were prepared according to standard methods. Ultrathin sections were made on the UMPP-7 Ultratome (Ukraine), stained with toluidine blue, contrasted with lead citrate and uranyl acetate. Ultrathin sections were examined under a Selmi Electron microscope (Ukraine) at an accelerating voltage of 125 kV.

Morphological examination of semi-thin sections was carried out using an OLYMPUS CX31 light-optical microscope with an OLYMPUS C-5050Z digital camera. The area of sections, the area of cells and their number in the dermis of biopsy specimens were determined using ImageJ Software with the magnification of the objective lens of 40x and the magnification of the ocular lens of 10x. The measurements were made 50 times in each group.

The obtained digital data (expressed in pixels) were converted into μm by dividing pixels by a coefficient specifically established for this purpose: 6379251 for a 10x lens and 98911797 for a 40x lens. Statistical data analysis was performed using the licensed software MS Office Excel 2007, analytical platform of STATISTICA Enterprise application (StatSoft Inc., USA), and capabilities of STATGRAPHICS (v 5.1, Microsoft, USA). The arithmetic mean and the standard error of the mean were calculated. The Mann-Whitney test with a significance level of 0.05 was used to compare the two groups. Comparison of the area occupied by the cells in the biopsy specimens of the experimental group was performed in relation to the control group or within the groups on a percentage basis.

RESULTS

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 wound model. In our previous report, we described a picture of scar biopsy specimens both without treatment and after transplantation of the dermal equivalent with xenogenic fibroblasts after staining of sections with hematoxylin and eosin and by the Weigert-Van Gieson method under the light microscope [10]. The structure of the developing epidermis, collagen formation and angiogenesis in granulation tissue were reported.

In the present work, we studied the ultrastructural organization of granulation tissue cells in the central and peripheral parts of the scar biopsy specimens from the control and experimental groups.

The electron diffraction patterns revealed signs of extracellular edema, mainly localized around active fibroblasts with multiple processes, at the periphery of the biopsy specimens of the control group. Active interaction of fibroblasts with macrophages was well visible. Lamellipodia were formed in the cell-cell contact zone. Longitudinal and transverse sections of collagen protofibrils could be seen in the intercellular substance. There were continuous blood capillaries with basal membrane swelling and fibroblasts surrounding the vessels. Accumulating residual bodies and swelling of the nucleus were observed in all the cells. There were active small lymphocytes with well-defined nuclear pores and extended granular ER near the nucleus, which is evidence of active biosynthesis of proteins.

Neutrophils and macrophages were often found in the central areas of the biopsy specimen in granulation tissue next to active fibroblasts, which indicated that the inflammatory reaction was not completely overcome. Extracellular matrix was edematous and contained collagen protofibrils. The nuclei of fibroblasts had deep invaginations and well-developed granular ER, providing high synthetic activity of these cells.

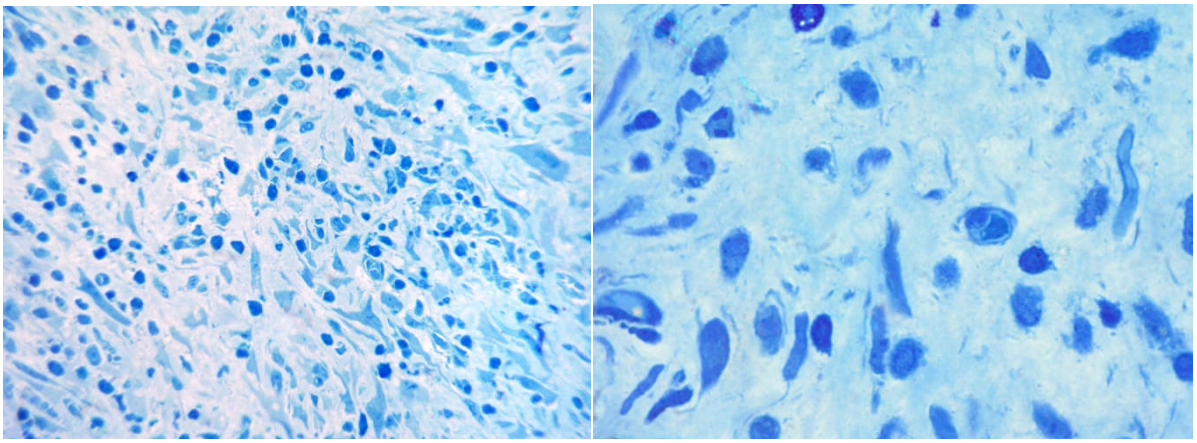
There was a significant difference in the size and number of cells between the central and peripheral regions in semi-thin sections of biopsy specimens of the control group. The area occupied by the cell in the central part was, on average, larger than the area occupied by the cell at the periphery by 10.53% (Table 1). The total average area occupied by the cells in the center was 13.64% less than at the periphery.

Table 1 : **The area of the cell and the average area of all cells in the biopsy specimens of the mice in the control and experimental groups**

Granulation tissue in the dermis	Periphery of biopsy specimens		Centre of biopsy specimens	
	The average area of the cell in microns	The average area of the cell in %	The average area of the cell in microns	The average area of the cell in %
Control group	48.37±0.14	12.41±0.05	54.06±0.12	10.92±0.10
Experimental group	43.42±0.12	10.88±0.02	49.77±0.15	9.88±0.02

In the mice of the experimental group, the silicone ring fell away from the healing wound by day 11.2±0.10 after transplantation of the dermal equivalent with allofibroblasts.

There was an increase in the number of cells in the semi-thin sections of biopsy specimens of the experimental group in both the centre and the periphery (Fig. 1A and Fig. 1B). At the same time, the average area of the cell was reduced by 11.40% in the periphery and by 8.61% in the centre compared with the control group.



A B

Fig. 1. Mouse skin biopsy specimens after transplantation of dermal equivalent with allogenic fibroblasts (experimental group). Colouring with toluidine blue (semi-thin sections). Magnification × 1000. A – the peripheral part of the biopsy specimen. B – the central part of the biopsy specimen.

The average area of all cells was reduced by 10.46% in the periphery and by 10.52% in the centre compared to the untreated group. At the same time, there was an imbalance in the average area of the cell and the average area of all the cells between the centre and the periphery after transplantation of the dermal equivalent due to a greater number of cells in the periphery. It can be explained by the natural ability of the wound to heal from the periphery to the centre. At the same time, this process was more

active after the wound closure with a dermal equivalent with xenogenic fibroblasts, which stimulated active collagen formation in granulation tissue.

In the central regions of the biopsy specimens of the mice in the experimental group, against the background of transplantation of a dermal equivalent with xenogenic fibroblasts, there was a well-developed granulation tissue with an intercellular substance, characterized by the predominance of a structureless fine matrix over the formed fibrillar component. At that, fibrils tended to be located mostly in the zone adjacent to the processes of fibroblasts. Oedema was absent.

There were macrophages with morphological signs of high functional activity: numerous long, curved and thin pseudopodia, "worm-like" invaginations in the cytolemma, abundance of complex endosomes/heterolysosomes. The nucleus contained euchromatin, heterochromatin and nuclear pores, the nucleolus being visible.

Active fibroblasts constituted a predominant pool of cells in the centre of the biopsy specimen and were localized, among other places, near the blood capillaries. Their nuclei were elongated, with a predominance of euchromatin and a lot of nuclear pores; granular ER and mitochondria were actively developed; there were few residual bodies. Many collagen protofibrils were visible. The stages of fibrillogenesis (intracellular and extracellular) could be seen. Multiple pinocytotic vesicles and protrusions were observed in capillary endotheliocytes, which indicated the continuous type of blood capillary and high metabolism in the area of fibrillogenesis.

We revealed a binucleated myofibroblast, which was in close contact with the plasma cell (Fig. 2). There were intracellular actin filaments in the composition of the myofibroblast and extracellular thin fibrils outside it. Profiles of granular ER were narrow, with local extensions, filled with homogeneous content with average electron density. Large endosomes were visualized in the cytoplasm of the myofibroblast. The plasmocyte contained well-developed granular ER and the Golgi complex. On the surface of the plasma cell facing the myofibroblast, there were numerous small drop-shaped protrusions not reaching the myofibroblast and long cytoplasmic processes in contact with it. The extracellular matrix was characterized by localized sites of fibrillogenesis. A predominance of euchromatin indicated a high level of biosynthetic processes in both the fibroblasts and the plasma cell.

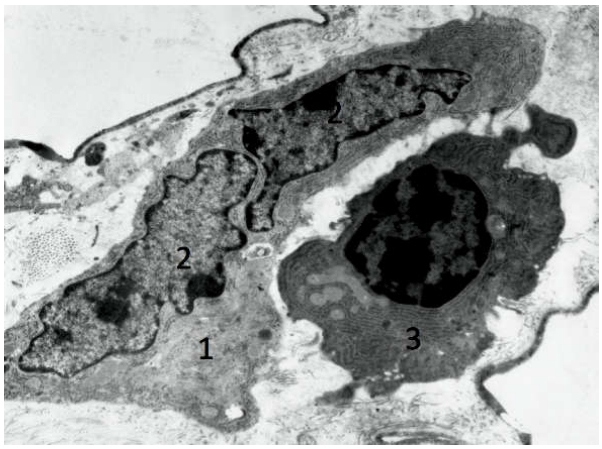


Fig. 2. Myofibroblast (1) with two nuclei (2) and the plasma cell (3) in the central part of the biopsy specimen after transplantation of the dermal equivalent with allogenic fibroblasts (experimental group). Electron micrograph. Magnification ×4000

At the periphery of biopsy samples, a well-developed granulation tissue in the initial stage of remodeling was also found. Numerous myofibroblasts were located in the zones of ordered arrangement of the fibrils. There were a lot of vacuoles, phagolysosomes and lysosomes in the cells.

Coated pits at the stage of vesicle formation, heterophagolysosomes at the initial stage of fusion of the content of the hydrolase vesicle with the substrate, residual bodies, lamellopodia indicated the resorption of the extracellular matrix of areolar tissue.

Granular ER was clearly visible in the processes of myofibroblasts; the cavities included, in some cases, not only a structureless matrix, but also a fibrillar component. There were also cases of rupture and direct contact of the content of vacuoles with the intercellular substance. Degradation of some cells dying by necrosis could be discerned.

The number of macrophages decreased gradually. Electron micrographs show the macrophages with signs of dysfunction: fragmentation of heterochromatin with perimembrane localization (the pre-apoptotic stage), deep invaginations of the karyolemma, numerous electron-lucent vacuoles of various sizes occupying more than half of the cell area. There were also macrophages at the stage of phagocytosis during resorption of the extracellular matrix. The characteristic features of sites of the intercellular substance surrounding capillaries were a prevalence of the amorphous component and weak organization of the fibrous component. Capillaries are filled with structureless content. Endotheliocytes showed signs of high resorption activity. Numerous protrusions were directed to the lumen of the vessel (they created a "mosaic structure" in the thick part of the cells in the cross section); there were pinocytotic vesicles on the basal side.

DISCUSSION

Variability and molecular remodeling are key mechanical features of ordinary connective tissue, in which collagen and other molecules of the intercellular substance can stretch, slide and undergo stable reorganization relative to each other [11]. The dermal equivalent is a complicated tissue-engineering construct based on collagen. Such a 3D structure, contrary to a 2D collagen layer, has rigidity of its own and architecture of collagen fibres which makes it possible for associated cells to attach in space and change topography of the construction to improve homeostasis. It is dominated by fibroblasts with leading dendritic extensions, migrating throughout dermal equivalent. Intercellular contacts are formed between the processes of fibroblasts, creating a network of cells similar to bonds between osteoblasts in bone tissue [12]. In this case, the effect of transplanted allogenic fibroblasts on the formation of granulation tissue can be the most bizarre and, as recent studies have shown, very effective in terms of the speed and effectiveness of healing ischemic wounds [7]. Emergence of binucleated fibroblasts with signs of high functional activity in the mice is a very interesting fact not previously described. Binucleation might be an illusion due to the deep invagination of the karyolemma, which is a gap, but in this case it is also a sign of a very high functional cell activity, provoked by transplantation of the dermal equivalent with xenogenic fibroblasts. A number of researchers agree that when organs and tissues are damaged, there is such a phenomenon as plasticity, consisting in fusion of mesenchymal stem cells with cells of the target organ [13]. Cellular plasticity is recognized as a fundamental feature of tissue biology and can be crucial for the survival of an organism. Recent studies have revealed heterogeneity and plasticity of dermal fibroblasts within the skin, which is important for tissue engineering [14]. It is also likely that transplanted allogenic fibroblasts do not merge with autologous fibroblasts independently, since it is known that to develop plasticity in vivo, certain factors are secreted by the damaged organ mobilizing stem cells (i.e., causing the release of stem cells from their natural niches in the body into the bloodstream) and contributing to their migration towards the damaged organ and its subsequent colonization. According to such concepts, one of these factors is probably SDF-1 secreted by allogenic fibroblasts [15]. Binucleated fibroblasts with an incompletely formed intercellular substance in the central zone of the biopsy specimen are highly active, most likely due to tetraploidy. Numerous experiments have proven that substrate adhesion is fundamental to complete cytokinesis in untransformed fibroblasts. When dividing, such fibroblasts in suspension cannot complete cytokinesis, therefore binucleated cells with the doubled number of chromosomes develop [16]. Binucleated cells retain an inactive state of p53 and are capable of intensively secreting components of the intercellular substance into G1 and S phase. However, binucleated cells arrest in G2, accumulate p53 and cannot start mitosis since tetraploid metaphases are not registered after one cell cycle period [15]. It is remarkable, that after being preserved in the G2 phase of the cell cycle, most binucleated fibroblasts become aging [17]. Inhibition of active functioning and entry into aging after cytokinesis can be an important mechanism to control the growth of granulation tissue and subsequent scarring.

CONCLUSION

On the 12th day after reparation of the model ischemic wound in all the groups, the granulation tissue of the peripheral and central regions differs considerably in its ultramicroscopic structure. Significant differences in the amount and size of cells, as well as the area they occupy, have been revealed. After transplantation of the dermal equivalent with allogenic fibroblasts, the number of cells increases, and the average area of the cell and that of all the cells decrease. In contrast to the control group, there are no signs of edema, no neutrophils and active degradation of macrophages in the granulation tissue of the central and peripheral regions, which indicates a low level of inflammatory response. Binucleated myofibroblasts with a high level of synthetic activity are found in the center of the healing wound. At the periphery of the

wound against the background of allogenic fibroblast transplantation, granulation tissue is in its initial stage of remodeling. Myofibroblasts prevail in the wound, providing its contraction.

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