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ORIGINAL ARTICLE

The Complex of Low-Molecular Hyaluronic acid and Dermal Fibroblasts changes the Gradient of SDF-1 secretion and the migration of MSC to a regenerating ischemic skin wound

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ABSTRACT
Cells of damaged tissues secrete Stromal-derived factor-1 (SDF-1), which is a chemoattractant for MSC with the CXCR4 receptor on the surface. The aim of the study was to study the expression of SDF-1 and CD34+ MSC in biopsies of regenerating model ischemic skin wounds after transplantation of low-molecular-weight hyaluronic acid (HA) and dermal allofibroblasts. The study was performed on 71 white Mature mice of the C57/B1 line at the age of 5-7 months, which were divided into CG and EG. A model ischemic wound was formed in both groups. For transplantation, a low-molecular weight 2% HA was used in combination with 1.33 million allofibroblast cells. The presence of SDF-1 and CD34-positive cells was determined by immunohistochemical method. It was found that the index of SDF-1-positive cells in the epidermis and dermis was higher in biopsies on the 4th day after tissue engineering design transplantation compared to the control, which attracts MSC. However, the index of SDF-1 + cells grows more slowly in the future, not reaching the values in KG, and begins to decrease earlier (by the 12th day in the epidermis and the 10th day in the granulation tissue). Similar dynamics is demonstrated by the index GMT. The presence of HA in the intercellular substance is characteristic of non-damaged tissue, which inhibits the production of SDF-1, which attracts less MSC. It is likely that the transplanted fibroblasts and subsequent epidermocytes themselves actively divide without the involvement of MSC and provide wound healing 16.94% earlier than in the control.
Keywords: SDF-1, GMT. CD34-positive cells, dermal allofibroblasts

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INTRODUCTION

Chronic skin wounds in an aging population against the background of hemodynamic problems, an increase in the number of cases of both diabetes and obesity are becoming a global health problem. Despite the increased prevalence, modern traditional therapies have limited effectiveness for accelerating wound closure and promoting healing [1]. In the case of skin damage, wound healing occurs in three overlapping stages: inflammation, proliferation, and remodeling [2]. When the healing process is chronicled, signs of all three stages may be present simultaneously. Skin dermal cells fibroblasts are crucial in all three phases, secreting components of the extracellular matrix, remodeling the extracellular matrix, and shrinking the wound [3]. Fibroblasts are used in regenerative medicine, being the main cells of skin substitutes created on the basis of cellular technologies. Skin substitutes can be based on intercellular components such as hyaluronic acid (HA), which create a microenvironment for fibroblasts. It is an unbranched glycosaminoglycan consisting of repeated disaccharide links of N-acetyl-D-glucosamine and D-glucuronic acid and is a non-sulfated glycosaminoglycan of the intercellular substance of connective tissue. GC is involved in many key processes, including cell signaling, wound repair, tissue regeneration, morphogenesis, and matrix organization, and has unique physical and chemical properties such as biocompatibility, Biodegradability, mucoadgesivity, hygroscopicity, and viscoelasticity [4].

Molecular weight and the circumstances of synthesis / degradation are the key factors determining the biological action of HA [5]. Hyaluronan with high molecular weight and low molecular weight demonstrate opposite effects [6].

Skin wound cells secrete stromal factor-1 (SDF-1, also known as CXCL12), which is one of the chemoatractants for mesenchymal stem cells (MSC) that have CXC chemokine receptor 4 (CXCR4) on the surface and binds to SDF-1 [7]. Mesenchymal stem cells (MSC) are an important source for repairing damaged tissues, both in animal models and in human clinical trials [8]. Migration of MSC to the target site is crucial in regenerative medicine [9]. There is no information about the expression of SDF-1 cells, as well as the presence of MSC in tissues of regenerating skin defects on the background of ischemia after transplantation of a construct from low-molecular HA and dermal allofibroblasts, thus determining the relevance of the study.

The aim of the study was to study the expression of Stromal-derived factor-1 (SDF-1) and the content of CD34+ mesenchymal stem cells in biopsies of regenerating model ischemic skin wounds after transplantation of low-molecular HA and dermal allofibroblasts.

MATERIAL AND METHODS

The study was performed on 71 white sexually mature mice of the C57/B1 line at the age of 5-7 months, which were kept in the vivarium of the Medical Academy named after S.I. Georgievsky. The animals were divided into control (CG) and experimental groups (EG). The distribution of animals by group is shown in table 1 (table 1). The experiments were conducted in accordance with all the principles of humanity included in Directive 2010/63/EU and in accordance with the ICMR guidelines for animal research (2006).

Table 1. Distribution Of Mice By Timing Of Material Collection In The Control And Experimental Groups

Time period after surgery	Control group (individuals)	Experimental group (individuals)
4 days	3	3
7 days	4	5
10 days	3	5
12 days	5	6
15 days	4	5
19 days	5	7
23 days	3	5
26 days	4	4
Total	31	40
Total in the study – 71 individuals		

Surgically, an ischemic skin wound was formed in the inter-scapular region of both groups of mice. In EG, dermal fibroblasts were obtained by fermentation and cultured in DMEM/F12 (Lonza) [10]. For transplantation, a low-molecular weight 2% HA of biosynthetic origin "Hialuron 2", manufacturer: Toskani Cosmetics (Spain) was used in combination with 1.33 million cells of allogeneic fibroblasts of 2-3 passages with the phenotype CD44+CD90+CD105+CD73+CD 45+CD31-CD34 - CD45-on the growth medium DMEM/F12. The mixture was introduced by tunnel method using needles 30G, 13mm.

On the 4th, 7th, 10th, 12th, 15th, 19th, 23rd and 26th day after surgery in both groups, the resulting scab or scar was intraoperatively excised and fixed in a 10% buffered formalin solution. The material was poured in paraffin and painted with H&E. Histological preparations were studied using the light-optical microscope OLIMPUS SX-31. The presence of SDF-1 and CD34-positive cells was determined by immunohistochemical method. The primary antibodies were SDF-1-polyclonal antibodies (Gene Tex Inc., USA) and CD34 - monoclonal antibodies (clone EP373Y) (Abcam, USA) in 1:100 dilution. Universal antibodies (HiDef Detection™ HRP Polymer system, "Cell Marque", USA) were used as secondary antibodies that allow detecting mouse and rabbit primary antibodies conjugated with an enzyme complex based on horseradish peroxidase. Control studies were performed for each marker in order to exclude pseudopositive and pseudonegative results. The sections were colored with Mayer's hematoxylin to visualize the nuclei. The index of SDF-1 and CD34-positive cells was determined by counting their number per 100 cells during microscopic examination (increase x 1350), followed by calculating the average percentage based on the results of the studied sections of each biopsy sample in CG and EG. The data obtained as a result of the calculation was processed using the computer program SPSS 7.5 for

Windows statistical software package (IBM Analytics, USA). In statistical processing, the following nonparametric criteria were used to assess the reliability of differences in mean values between groups: u-the Mann–Whitney test (the probability value P = 0.05 was considered statistically significant), H-the Crascall-Wallace test.

RESULTS

On the 4th day after the operation, there is no epidermis in the sections of biopsies of both groups of mice stained with hematoxylin and eosin. In CG, a huge thickness scab of inflammatory cells, cellular debris and exudate makes the skin defect. In EG, an extensive scab also closes the skin wound, under which there are oxyphilic-colored cavities filled with low-molecular-weight ha. At the border with the underlying tissues, a small accumulation of damaged cells of the lymphocytic series is traced. The entire skin defect in mice of all groups is made by white adipocytes that have risen from the hypodermic. Collagen fibers and capillaries are extremely small and almost non-existent.

SDF-1-positive cells are present in the biopsies of both experimental groups (table 2). In biopsies, the CG of such cells directly under the scab (conventionally called the epidermis) is 3 times less 71.18+0.18%, and 37.45+0.18%, respectively, higher than in the control. MSC expressing the CD34 marker are present only in the deep layers of EG biopsies.

On the 7th day, the silicone ring holding the edges of the wound is still tightly fixed. In control, the wound is covered from the outside with a scab consisting of dead cells, under which a partial epithelization of the wound is detected on the sections: there is an epidermis consisting of 1-2 rows of cubic epithelial cells. After transplantation of allofibroblasts associated with low-molecular HA, the epidermis has up to 3 rows of cubic epidermocytes. A statistically significant increase in the index of SDF-1 – positive epidermocytes, which provide chemoattractant secretion for MSC, continued in the biopsies of both groups (see table 2). Under the epidermis, the resulting granulation tissue is localized, with a small number of capillaries, fibroblasts and collagen fibers. The index of SDF-1+ cells of non-differentiated granulation tissue from day 4 to day 7 increased by 25.64+0.20% in CG and by 40.39+0.15% in EG. At the same time, the index of such cells in the EG is 47.43+0.20%, more. The presence of MSC (CD34-positive cells) is still detected only in the granulation tissue of the EG.

Table 2. Index Of Sdf-1-Positive Cells And Cd34-Positive Cells In Skin Wound Biopsies Of The Control And Experimental Groups

Day after the operation	Control group				The experimental group			
	Index of SDF-1+ cells in %		*CD34+ cell index in %*		*Index of SDF-1+ cells in %*		*CD34+ cell index in %*	
	EP	GT	EP	GT	EP	GT	EP	GT
4-e	3,21±0,01	10,22±0,07	0	0	11,14±0,11*	16,34±0,13*	0	6,15±0,01*
7-e	12,41±0,11**	14,41±0,11**	0	0	18,28±0,10**	27,41±0,14**	0	13,27±0,10**
10-e	20,00±0,12**	19,38±0,11**	0	5,10±0,01**	22,33±0,12**	36,82±0,15**	0	24,72±0,11**
12-e	70,67±2,01**	26,51±0,13**	1,23±0,01**	12,18±0,09**	113,42±0,13**	29,56±0,12**	6,24±0,01**	19,33±0,14**
15-e	76,23±0,22**	31,58±0,18**	2,12±0,01	19,16±0,11**	5,04±0,09**	12,27±0,11**	2,51±0,01**	10,47±0,11**
19-e	72,33±0,22	29,27±0,20**	2,12±0,01	10,09±0,05**	0**	3,17±0,02**	0**	2,82±0,05**
23-и	39,15±0,14**	16,10±0,11**	0**	3,10±0,02**	0*	0**	0	1,02±0,01**
26-e	0**	0**	0	1,02±0,01	0	0	0	0**

* Statistically significant differences from the control, P = 0.05.

** Statistically significant differences from the previous day, P = 0.05.

On the 10th day, EG mice do not have a silicone ring. Epithelization of the wound and spontaneous fall of the ring was recorded at 10.3+0.10 days after the operation. In CG, the ring is still sewn to the edges of the skin defect. A scab that has decreased in thickness closes the wound in both groups, but the scab in control is much thicker, and the epidermis is thinner. The SDF-1+ epidermocyte index continued to grow in CG and EG and amounted to 37.95+0.19% and 18.14+0.16%, respectively. Such epidermocytes in the EG are 10.43+0.17% more than in the control group. On the 10th day of wound repair, CD34-positive cells were not found in the epidermis of both groups. The formation of granulation tissue in wound biopsies

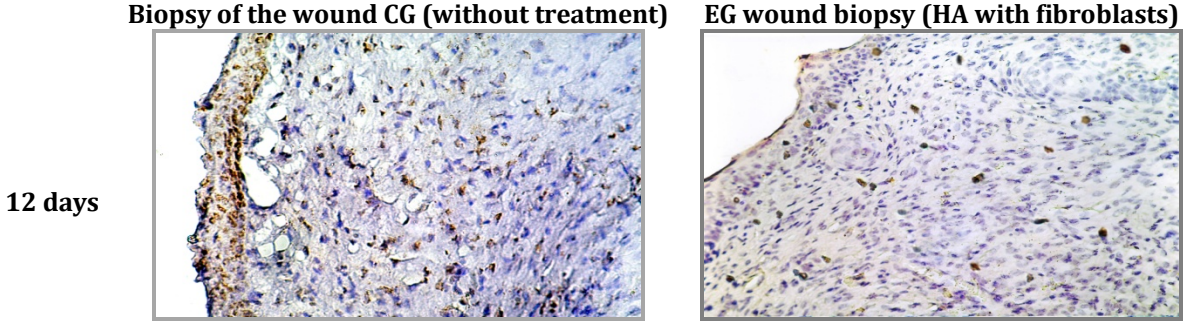
was most significantly advanced in the EG, where a network of collagen fibers was formed, between which lie fibroblasts and blood capillaries. By day 10, the index of granulation tissue cells with SDF-1 expression increased by 25.64+0.20% in CG and by 25.56+0.10% in EG, remaining by 47.37+0.13%, more than in the control. At this time, individual CD34-expressing cells were detected for the first time in the granulation tissue of KG. In the granulation tissue of EG biopsies, the MSK index increased by 46.32+0.19% compared to 7 days. Their index is much higher than that in CG (79.37+0.20%).

In CG mice, spontaneous fall of the silicone ring was recorded at an average of 12.4+0.10 days after the operation to create a model wound due to its epithelization and suture eruption, which is 16.94% later than in EG.

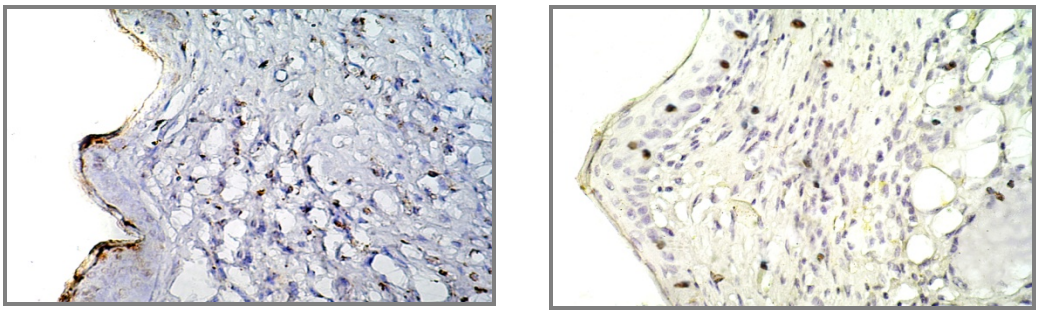
On the 12th day in CG under the thick remnants of the scab, full epithelization of the wound was detected. The entire skin defect is filled with developing granulation tissue, which contains cells of tissue and hematogenic origin. The papillary and reticular layers of the dermis are not differentiated and are formed by evenly randomly localized thin collagen fibers that form a reticular structure. Between the collagen fibers there are cells represented mainly by functionally active fibroblasts. The surface of the wound in the EC is covered with the epidermis throughout. The scab is completely absent. The appearance of the papillary layer of the dermis in the form of a wavy border between the basement membrane of the epidermis and the granulation tissue to be treated has been observed. The number of epidermocytes with the SDF-1 marker in CG biopsies is very large, the increase in their index from the 10th to the 12th day of wound healing was 71.70+0.26% (figure 1). The index of SDF-1-positive epidermocytes in the EG decreased by 85.74+0.23%, which made it 81.01+0.06 % less than in the control. In the epidermis of CG and EG, there are single MSC. Granulation tissue in the EG is characterized by the appearance of initial signs of fibrosis: oxyphilic painted bundles of collagen fibers have an acquired parallel orientation. From the 10th to the 12th day, the index of SDF-1+ granulation tissue cells in CG continued to increase and increased by 26.90+0.22%. The opposite process was observed in biopsies of granulated EG tissue: the index of SDF-1 + cells decreased by 19.72+0.19 %, which led to the fact that the index of cells with such expression in EG became 10.32+0.05 %, less than in the control. The index of cells with CD34 antigen increased in CG by 58.13+0.23%, and in EG decreased by 21.80+0.12 %. This did not lead to a change in the ratio of the MSK index in the groups: in the EG, there were still more of them by 36.99+0.16% than in the control.

On day 15, the experimental ischemic wound in mice is located on the path of scarring more pronounced in the EG, where the collagen fibers lose their mesh structure and are located in thin bundles parallel to the epidermis. The index of SDF-1-positive cells in CG increased by 16.05+0.14% compared to the previous period. In EG, the index of such granulation tissue cells decreased by 58.49+0.23%. In General, on day 15, the index of SDF-1 + cells in the EG is less by 61.15+0.22 % than in the control. The MSK index decreased in the granulation tissue of biopsies of both groups: in CG by 47.34+0.33%, in EG by 45.84+0.20%. On day 15, the index of CD34-positive cells in the EG is 45.35+0.12% less than in the control. In the epidermis during this period, the index of CD34-positive cells in CG and EG is the same, although in the epidermis of CG it increased by 49.98+0.22 %, and in EG it decreased by 59.78+0.20%.

Figure 1. Results of immunohistochemical study of stromal factor-1 marker expression (SDF-1) in biopsies of regenerating ischemic wounds.



15 days



Immunohistochemical study with SDF-1 antibodies, hematoxylin staining of the nuclei, zoom×400.

In the subsequent period of up to 26 days, the wound process in the biopsies of both groups was at the stage of scar formation. This process was most actively developed in the cells of EG biopsies, which was accompanied by the termination of the secretion of the MSK SDF-1 chemoattractant (see table. 2) and the preservation of single MSC only in deep layers of scar tissue. In KG, this process is delayed.

DISCUSSION

Skin wounds produce a variety of chemokines, such as CXCL12 (SDF-1), which can serve as chemoattractants for MSC [11]. MSC are adult stem cells that have a therapeutic effect for chronic wounds and can differentiate into active fibroblasts [12]. We found that on the 4th day after transplantation of allofibroblasts in combination with low-molecular weight ha, the index of SDF-1+ and CD34+ cells is higher than in KG. In the future, the index of SDF-1 + cells in CG actively increases, and in EG increases slowly, because the presence of HA in the intercellular substance is characteristic of non-damaged tissue and, apparently, this inhibits the production of SDF-1+. MSC are not actively involved in EG, so there are relatively few of them, although the wound heals faster than in KG. Probably, the transplanted fibroblasts themselves actively divide and form granulation tissue. Their proliferative potential is sufficient even without the involvement of MSC. In KG, a high concentration of SDF-1 is created, but the arrival of MSC is delayed and the wound healing is prolonged for a longer period. Grigoryan A. S. [13] showed that, despite the fact that cells of damaged tissues secrete a large amount of SDF-1 factor; its excess amount not only does not attract MSC, but also repels them. There is also evidence that the medium in the damage zone is rich in proteolytic enzymes: serine proteases, catepsin G, elastases and matrix metalloproteinases, which destroy chemoattractants such as SDF-1 [14].

CONCLUSION

Thus, we obtained that the index of SDF-1-positive cells in the epidermis and dermis was higher in biopsies of a healing model ischemic skin wound on the 4th day after allofibroblast transplantation in combination with low-molecular weight HA in comparison with the control, which attracts MSC. However, the index of SDF-1 + cells grows more slowly in the future, not reaching the values in KG, and begins to decrease earlier (by the 12th day in the epidermis and the 10th day in the granulation tissue). Similar dynamics is demonstrated by the index GMT. The presence of HA in the intercellular substance is characteristic of non-damaged tissue, which inhibits the production of SDF-1, which attracts less MSC. It is likely that the transplanted fibroblasts and subsequent epidermocytes themselves actively divide without the involvement of MSC and provide wound healing 16.94% earlier than in the control.

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