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Macrophages as homeostatic regulators in the ischemically damaged myocardium after use of allogenic biomaterial

Lebedeva A.I.[1], Muslimov S.A.[1], Gareev E.M.[1], Afanasiev S.A.[2], Condratyeva D.S.[2], Popov S.V.[2]

[1] *Russian Eye and Plastic Surgery Centre*
67/1, R. Zorge Str., 450075, Ufa, Russian Federation

[2] *Research Institute of Cardiology, Tomsk National Research Medical Centre (NRMC)*
Kievskaya Str., 111a, Tomsk, 634012, Russian Federation

ABSTRACT

Macrophages as the effector cells play a key role in initiating the inflammatory process and predetermine the manifestation of the postinfarction cardiosclerosis. The population of these cells is heterogenous and is mainly represented by M1 and M2 phenotypes. Alloplant biomaterial (ABM) is resorbed by the macrophages which became the regulators of the cellular interaction in tissues.

The aim of the investigation was to reveal the peculiarities of the postinfarction healing of the myocardium following the ABM insertion and to assess the population change in the dynamics of macrophages and c-kit+ cells.

Materials and methods. The experimental investigations were carried out on 100 male Wistar's rats weighing 0.18–0.25 kg. All the animals had coronary occlusion by way of ligating the arteries. In the experimental group, the ABM (12 mg) suspension was intramyocardially administered simultaneously with the vessel stricture formation. The harvesting of hearts was carried out at 3, 7, 14, 30, 45 days.

Results. In the experimental group the course of the inflammatory process was characterized by the onset of the early proliferative stage, whereas in the control group colliquative necrosis was developing. It was caused by different degrees of the macrophage reaction expression. The number of CD68+ cells in the rat reactive zone of the control group was bigger than in the experimental one. In the experimental group the ABM-induced macrophages of mesenchyme origin were revealed and c-kit+ cells were considerably more in number than in the control one. After 45 days, the scar area index in the experimental group was significantly less than in the control group.

Conclusion. ABM had a histoprotective effect under the conditions of the acute myocardial ischemia due to the inhibition of macrophage migration and induction of cellular cardiomyogenesis.

Key words: alloplant biomaterial, scar area, myocardium.

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✉ *Lebedeva Anna I.*, e-mail: Jeol02@mail.ru.

Макрофаги как регуляторы гомеостаза миокарда после его ишемического повреждения в условиях применения аллогенного биоматериала

Лебедева А.И.[1], Муслимов С.А.[1], Гареев Е.М.[1], Афанасьев С.А.[2], Кондратьева Д.С.[2], Попов С.В.[2]

[1] *Всероссийский центр глазной и пластической хирургии*
Россия, 450075, г. Уфа, ул. Р. Зорге, 67/1

[2] *Научно-исследовательский институт (НИИ) кардиологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук*
Россия, 634012, г. Томск, ул. Киевская, 111а

РЕЗЮМЕ

Ведение. Макрофаги как клетки-эффекторы играют ключевую роль в инициации воспалительного процесса, предопределяют выраженность постинфарктного кардиосклероза. Популяция этих клеток является гетерогенной и представлена преимущественно фенотипами М1 и М2. Аллогенный биоматериал аллоплант (БМА) резорбируется макрофагами, продукты резорбции влияют на их способность регулировать клеточные взаимодействия.

Цель. Раскрыть особенности постинфарктного заживления миокарда после введения БМА. Оценить динамику изменения численности макрофагов и c-kit$^+$-клеток.

Материалы и методы. Экспериментальные исследования были проведены на 100 самцах крыс линии Вистар массой 0,18–0,25 кг. Всем животным была проведена коронароокклюзия верхней трети левой нисходящей коронарной артерии. В опытной группе сразу после стенозирования артерии в ее бассейн интрамиокардиально вводили суспензию, содержащую 12 мг БМА. Использовали гистологические, электронно-микроскопические, иммуногистохимические (CD 68, c-kit, Timp-2), морфометрические и статистические методы исследования. Забор сердец проводили через 3, 7, 14, 30, 45 сут.

Результаты. В опытной группе течение воспалительного процесса характеризовалось наступлением ранней пролиферативной стадии, в то время как в контрольной развивался колликвационный некроз. Группы характеризовались различной степенью выраженности макрофагальной реакции. Число CD68$^+$-клеток в реактивной зоне в контрольной группе было больше, чем у опытной группы. Напротив, в опытной группе выявлены БМА-индуцированные макрофаги мезенхимного происхождения, а численность c-kit$^+$-клеток была значительно больше, чем в контроле. Спустя 45 сут индекс площади рубца в опытной группе был статистически значимо меньше, чем в контроле.

Заключение. БМА в условиях острого ишемического поражения миокарда оказывал гистопротекторный эффект за счет ингибирования миграции макрофагов и индукции клеточного кардиомиогенеза.

Ключевые слова: биоматериал аллоплант, площадь рубца, миокард.

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INTRODUCTION

In the course of experiments using alloplant biomaterial (ABM) it was established that the key histion cells during the regeneration of fibrous connective and skeletal muscle tissue are macrophages with M1 phenotype. Their number significantly exceeds the amount of these cells in the control groups in which the defect infliction was not treated with the biomaterial in question [1, 2]. It was shown that the use of ABM had a positive effect upon the cardiac muscle condition and improved its structure following ischemic damage [3]. The ABM biodegrates into the tissue and its resorption products are the chemoattractant of the stem progenitor cells which induce the regeneration process [4, 5]. There are conflicting views on the negative role of M1 macrophages in the healing process of the ischemic damaged myocardium as key cells promoting cardiomyocyte damage, inflammation manifestation and fibrosis progression. Consequently, the study of macrophage involvement in inflammatory and degenerative processes developing in the cardiac muscle, following coronary occlusion experiments and when administering the ABM, appears relevant.

The aim of the study was to understand the effect of ABM on the post-infarction myocardium healing process and evaluate the dynamics behind the changing number of macrophages and c-kit+ cells.

MATERIALS AND METHODS

Experiments involving ABM were carried out on 100 male Wistar rats weighing 0.18–0.25 kg. All the animals were divided into two groups. The myocardium infarction modeling in the control group (n = 50) was performed as follows: all the animals under general anesthesia (intramuscular injection of Zoletil) underwent left-sided thoracotomy with further ligation in the upper third interventricular branch of the coronary artery (*r. interventricularis paraconalis a. coronarii sin.*). 12 mg of ABM suspended in physiological solution was administered into the cardiac muscle, in its pool zone, of the rats in the experimental group (n = 50) immediately after the coronary artery ligation. The ABM dose was chosen arbitrarily. The rats in both groups were euthanized after the experiment by lethal insufflation of ether vapors after 3, 7, 14, 30, 45 days. Ten rats were taken for each point of the study.

The studies were conducted according to the Rules of good laboratory practice of the Russian Federation in line with legislation adopted from the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasburg,1986). The studies were also carried out in accordance with the approved written protocol on standard operating procedures of a researcher as well as official laboratory guidelines on animal treatment and alternative models in biomedical studies [7].

Alloplant® biomaterial was developed in the Federal State Government-Funded Institution "Russian Eye and Plastic Surgery Centre" under the Ministry of Health of the Russian Federation, in the city of Ufa. This biomaterial is produced according to technical specifications 42-2-537-87; it is certified and was approved for clinical use by the order of the USSR Ministry of Health No. 87 901-87 dated 22.07.1987.

Histological study. The allogeneic biomaterial in this study was made from rat tendons and enlarged to a size of 50–80 mcm. The Ethics Committee approved the study protocol No. 31 dated 12.10.2015. For the histological investigation the hearts were fixed with 10% solution of neutral formalin, then dehydrated with increasing concentrations of alcohol and embedded in paraffin as per the generally accepted method. The sections were prepared with the use of LEICA RM 2145 microtome (Germany) and stained using the Mallory's staining technique.

Immunohistochemical study. The 4 mcm-thick paraffin sections were stained by Leica Microsystems Bond TM immunohistostainer (Germany). CD 68 and Timp-2 diluted in the proportion of 1 : 300 (Santa Cruz Biotechnology, USA) were used as primary antibodies. Single and double immunolabeling of cells for the given antibodies was carried out. An indirect Leica Bond (Novocastra TM , Germany) streptavidin-biotin detection system was used for unmasking. Assessment of reaction specificity when staining the sections was determined without primary antibodies. The positively stained cells were calculated in 20 fields of view of each specimen (n = 6) when magnified by X400. The investigation and visualization of the specimens was conducted with the use of the Leica DMD 108 (Germany) light microscope equipped with specialized software to manage settings and capture images.

Electron microscopic study. The myocardium pieces 1–2 mmi in size fixed by 2.5% glutaraldehyde solution were used for the electron microscopic study. The solution was prepared on

the cacodylate buffer (pH 7.2–7.4) with further post-fixation by 1% OsO_4 solution on the same buffer. The material was dehydrated in increasing concentrations of alcohol and embedded into Epon-812 according to the generally accepted method. EM UC7 (Leica, Germany) ultramicrotome was used to prepare semithin sections which were stained by toluidine blue solution based on 2.5% anhydrous sodium solution. Areas were chosen on the specimens for the electron microscopic studies. The ultrathin sections were contrasted by 2% water solution of uranyl acetate and of lead citrate according to Reynolds. They were studied by JEM-1011(Jeol Ltd.; Japan) transmission microscope.

STATISTICS

Each heart was cut into five sections to determine the size of the postinfarction scar. The scar area index (SAI) was measured in the specimens of the heart cross-sections using ITEM software in the following way: the ratio between the scar area and left ventricle wall area was multiplied by 100%. The analysis of SAI values was performed using non-parametric methods, namely, the univariate Kruskal – Wallis analysis of variance and comparison of uncorrelated data by Mann – Whitney method [8].

RESULTS

The difference in healing between the ischemic damaged myocardium in the control group and that in the experimental ones was significant. The SAI data in the experimental group insignificantly depended upon the follow-up periods ($\chi^2 = 5.7$, $p > 0.12$). However, the values of this parameter tended to reduce gradually. The distribution medians by the 7th day totaled 22.7%, (0%, 43.3%) and dropped significantly to 13.4% (0%, 22.2%). They decreased on the 14th day, whereas on the 30th and 45th days they reached 16% (0%, 32.1%) and 5.2% (0%, 33.8%) ($p = 0.14$ and $p = 0.02$, respectively). The difference between the 14th, 30th and 45th days turned out to be statistically insignificant ($p = 0.23 \div p = 0.75$).

In the control group, the dependence of SAI on follow-up time was also statistically insignificant ($\chi^2 = 6.3$, $p = 0.10$). The differences in the SAI level from the initial one (day 7) were statistically significant only on the 30th day ($p = 0.01$). Figure 1 shows that during the whole period of observation in the control group there were no cases of zero SAI values. Comparison of both experimental groups at different time periods of the study showed that at implantation of ABM at all follow-up periods, SAI was statistically significantly less than in the control ($p = 0.01 \div p < 0.0001$).

a *b* *c*

Fig. 1. Index in the experimental and control groups (*a*): on the abscissa axis – observation time (days). The ordinate of the index area of the scar (%): *b* – cross-section of the myocardium after 45 days in the control group, × 40; *c* – myocardial cross-section after 45 days in the experimental group, ×40. Mallory's staining

Macrophage cells are of great importance in fibrous progression and scar manifestation [6]. The number of CD68 macrophages in the control group within the reactive zone of the ischemically damaged cardiac muscle exceeded the values of the experimental group almost throughout the entire experiment. In the control and experimental groups, the recurring rise and subsequent fall of the cell number was, on the whole, highly significant ($\chi^2 = 76.3$, $p << 0.0001$ and $\chi^2 = 45.2$, $p << 0.0001$, respectively). The number of $CD68^+$ cells in the control group was statistically significantly greater than the number in the experimental group during the follow-up period from the

3th to 14th day ($p = 0.003$ and less). The remodeling attenuation process of myocardium and scar formation took place over a period from the 30th to 45th day. This caused a decrease in the number of macrophages in both groups ($p = 0.12$) on the 45th day and transformation from the exudative-proliferative phase of inflammation into the recovery stage (Fig. 2).

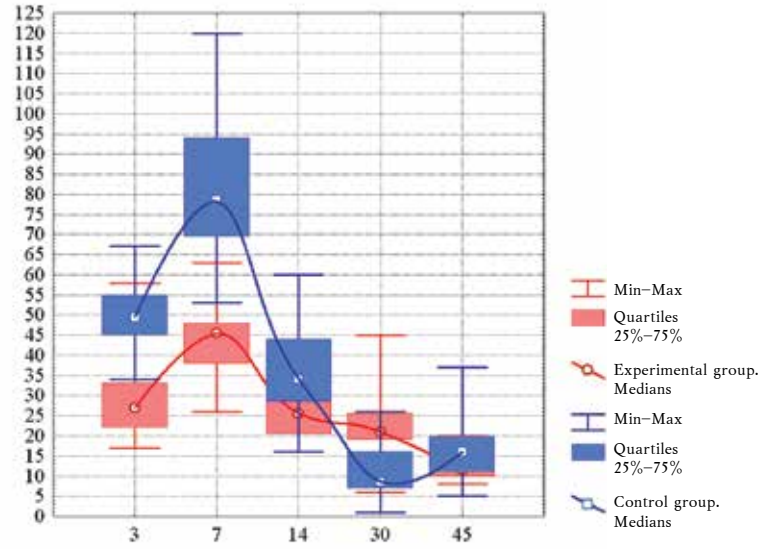
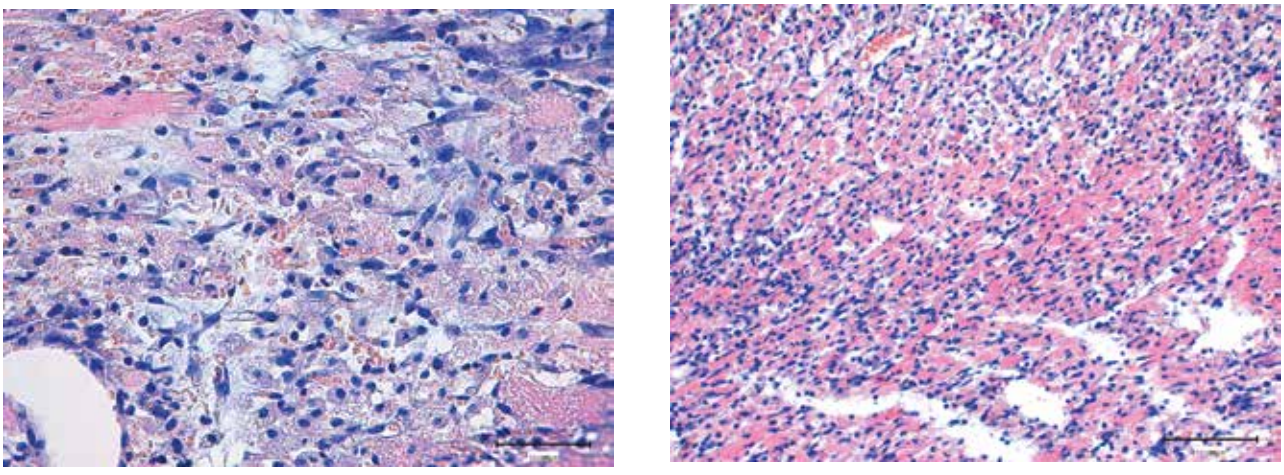


Fig. 2. The number of CD68+ macrophages in rat myocardium in experimental and control groups. On the x-axis is "days". On the ordinate axis – the number of cells

Through evaluation of the dynamics of pathomorphological changes, it was revealed that the initial stage of inflammation (day 3) was characterized by the early onset of the proliferative phase and formation of the granulation tissue in the perifocal area of the ischemically damaged myocardium. This is where thin collagen fibres, mesenchymal and macrophagal and fibroblastic infiltration were observed (Fig. 3, *a*).

In the control group, a wide cell shaft consisting of macrophages, lymphocytes, and neutrophils, was formed in place of the decaying cardiomyocytes. In this study, C-kit $^{+}$ cells in both groups were determined mainly in the peri-infarction and perivascular zones. Despite the autogenous origin of C-kit^{+} stem cells and absence of antigenicity factors they were subjected to phagocytosis by macrophages (Fig. 4, *a*).

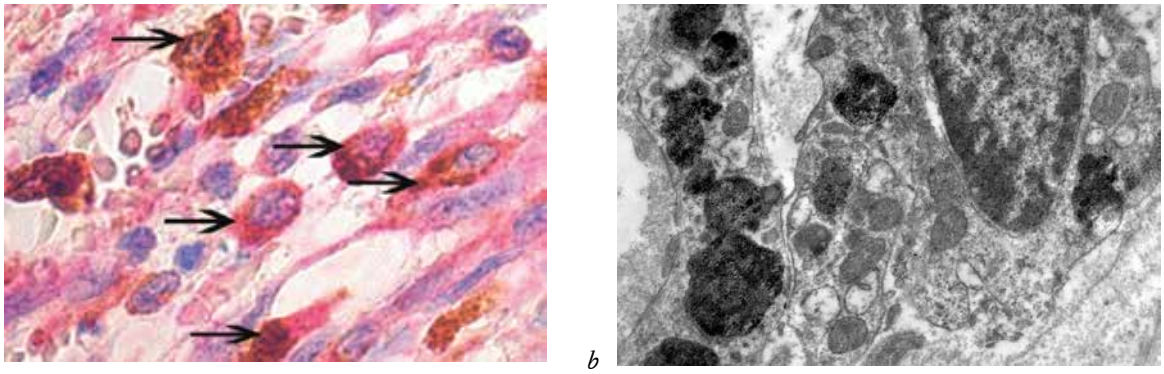


a *b*

Fig. 3. Morphological changes in the myocardium after 3 days: *a* – formation of granulation tissue in the perifocal zone, infiltration by macrophages, mesenchymal cells, fibroblasts in rat myocardium 3 days after coronary occlusion and ABM administration, *b* – macrophage-lymphocytic cell wall in the zone of necrotically changed cardiomyocytes 3 days after coronary occlusion. Stained with Hematoxylin and Eosin

Numerous macrophages phagocyting undifferentiated cells on the electron microscopic level were also recorded. Fragments of cytoplasm and pyknotic nuclei were detected in phagocytic vacuoles, and macrophage cells showed signs of activation. The nuclei were oval-shaped and contained large amounts of euchromatin; numerous large mitochondria with a darkened matrix and parallel oriented lamellar crystal were observed in the wide cytoplasma rim. The cytolemma formed deep invaginations. Golgi apparatus was well developed with piled up elongated flat cisterns and uncoupled vesicles (Fig. 4B).

When determining free C-kit+ cells, which were not subjected to macrophagal resorption, it was revealed that their number in the experimental group had surpassed statistically significantly the control group during the follow-up period (P< 0,001) (Fig. 5).



a *b*

Fig. 4. Phagocytic macrophages: *a* – CD68+/c-kit+ 7 days after coronary occlusion. CD68 (brown), c-kit (red). Chromogen granules are detected in the cytoplasm of macrophages. Double indirect immunoperoxidase method for antigen detection with Hematoxylin staining, ×600; *b* – Phagocytic macrophage with vacuoles of cellular detritus 7 days after coronary occlusion, ×10.000. Electronograms

Hale positive macrophages expressing Timp-2 were revealed in the zone of implantation in the subepicardial space (Fig. 6).

DISCUSSION

Numerous factors, one of which being macrophage reaction induced by ABM, contributed more favorably to myocardial infarction healing in the experimental group. It has already been proven that the products of ABM biodegradation turn into chemoattractants of monocytes and macrophages during the connective tissue healing followed by the inflammatory and destructive process and after inflicting damage [2]. Macrophage cells displayed regeneration efficiency as a result of full-fledged phagocytosis and regula-

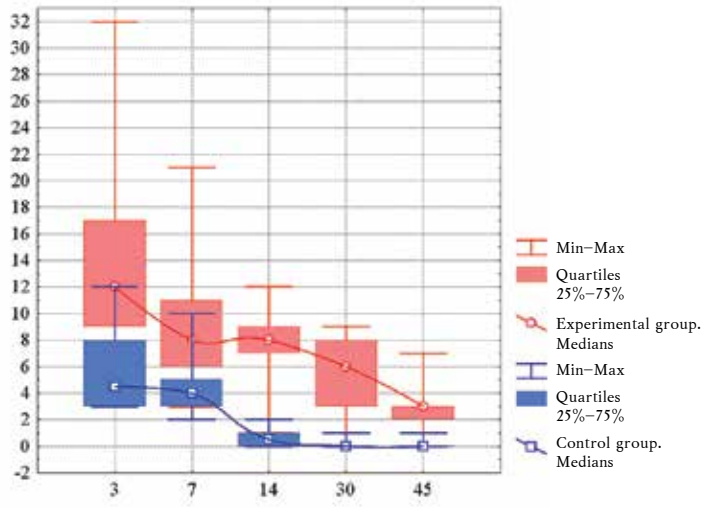


Fig. 5. The number of free c-kit+ cells in rat myocardium in the main and control groups, on the x-axis is “days”, on the ordinate axis – the number of cells

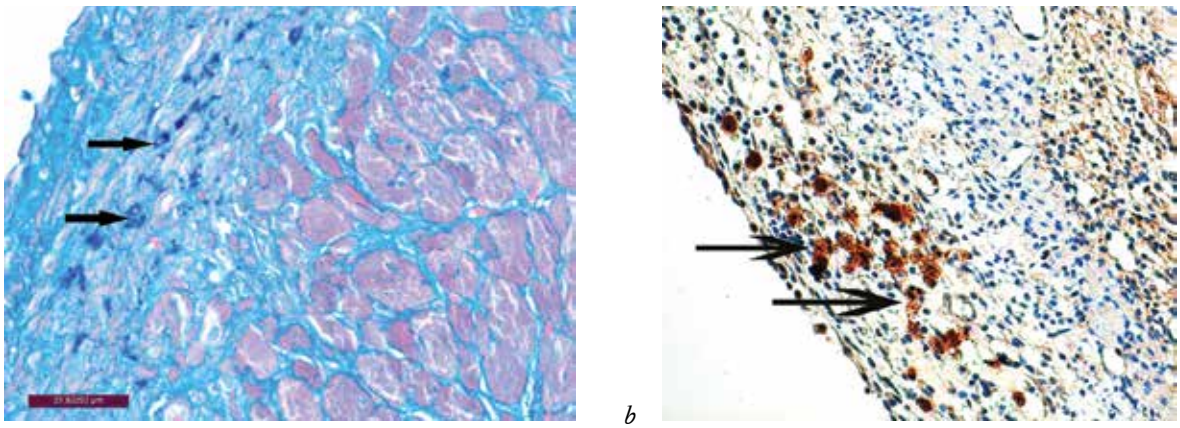


Fig. 6. Macrophages in subepicardial space after AMB administration after 7 days: *a* – GAG-positive macrophages (↑). Hale staining; *b* – Timp-2+ macrophages (↑). Indirect immunoperoxidase method for detection of Timp-2 with additional Hematoxylin staining, ×400

tion of the proliferative phase of the inflammation. They inhibited the fibroblastic activity by M1 stimulation of macrophages and prolongation of cytoxic phase [9, 10]. We got the opposite result in this study in case of acute myocardial infarction. Within 3 days, the ABM particles were resorbed and were not detected in the tissue. One can assume that after the biomaterial resorption, the ABM-induced macrophages became the regulators of intercellular interactions and stimulated the onset of the early proliferative inflammation phase activating fibroblastic cells.

In the control group the ischemically damaged cardiomyocytes initiated a succession of inflammatory cell reactions which resulted in increased inflammation, expansion of the damaged zone and scar manifestation. This observation was confirmed by the data of other researchers who had illustrated that peak levels of the corresponding family of proinflammatory (CD14+) macrophages and/or monocytes negatively correlated with the restoration of the left ventricle function following the acute myocardial infarction [11]. Dysregulated infiltration leads to the extension of myocardial infarction, expansion of the left ventricle and cardiac insufficiency. Monocytosis increases and extends stages of alteration and exudation due to the spectrum expression of inflammatory monokines (TNFa, IL1, IL6 etc) which, in turn, induce the exudative stress spreading over the nearby cardiomyocytes, thus expanding the necrosis zone. As a result of inflammation, the left ventricle remodeling is increased in case of the ischemic damages to the myocardium [12].

Macrophages are the polymorphic cellular population, the phenotype of which is determined by the microenvironment signals. In the experimental group after the use of ABM, the products of its biodegradation create a certain microenvironment, together with an anti-fibrogenic effect [9], which induces TIMP-2 expression by random cells. This phenomenon helps to decrease inflammation when acute ischemia occurs [13]. The modulation approach of macrophages changes according to their environment. It was revealed that phenotypes and functions of macrophages are formed by the corresponding organ microenvironment [14]. The transplantation of differentiated peritoneal macrophages into the pulmonary medium, for example, induced the transcriptional landscape reprogramming of those cells and their acquisition of new specific tissue functions [11, 15]. Thus, in case of acute ischemic myocardial damage, ABM has an anti-inflammatory effect and is a factor in the switching of the phenotype of macrophages from M1 to M2. Conversely, the destructive cardiomyocytes in the control group provoked a number of inflammatory reactions, due to the pronounced expression of metalloproteinases MMP-9 [13]. It is known that the regenerative process participants in the myocardium are not only effector fibroblast cells, but also cardiomyogenic progenitor cells. It is assumed that stem cell niches as well as epicardial cells, hematopoietic stromal cells etc. can be the source of stem cells [16,17]. The differentiation direction of progenitor stem cells is often unpredictable. This is explained by high probability of teratoma formation [18]. ABM stimulated migration of poorly

differentiated C-kit+ cells. Phagocytosis by macrophages of C-kit+ cells is probably connected with the genetically programmed mechanism of anti-tumorigenicity. In spite of this fact, the level of progenitor cells in the experimental group remained high enough which contributed to a more wholesome regeneration of myocardium and inhibition of scar tissue development.

Macrophages populate heterogenous cells.

Macrophages of mesenchymal origin, otherwise known as "matrix-forming", have also been identified during previous experiments with ABM [1, 19, 20]. They featured Vimentin+ /Hale+ / CD68+ /PCNA phenotype and secreted glycosaminoglycanes (GAG); this phenomenon being typical of fibroblast cells. Macrophages appeared to be of the mesenchymal origin. It was revealed in the study that they had expressed Timp-2 tissue inhibitor of metalloproteinase. Presumably these cells play a structural and informative role for cellular cooperation and create homeostasis in the inflammation focus. Their presence is connected with synthesis of the hydrocarbon component. Timp-2 has a histoprotective effect due to the anti-inflammatory mechanism in the myocardium [21] which can set in motion the early proliferative phase of inflammation and homeostasis regulation. The discovery of macrophages of the given phenotype is consistent with the observation that the adult human heart contains macrophages of embryonal origin capable of tissue restoration. It is worth noting that though these families are present in the resting adult heart, the tissue-resident macrophages are lost after injury of heart in adults and are substituted by inflammatory monocytic macrophages of the medullary origin [14]. Thus, ABM had a histoprotective effect in the case of acute ischemic myocardium. Differences in the number, composition and function of microphages contribute to varying models of restoration and remodeling of the left ventricle observed in the given experiment.

CONCLUSION

Coronary artery stenosis alongside with ABM use allows to reduce the myocardium scar area by 2.74 times.

The ABM use decreases myocardium infiltration by macrophage cells.

During the myocardium restoration after the ischemic damage, macrophages are capable of actively phagocytizing autogenic stem cells.

There exists a population of GAG-positive macrophages in the ABM implantation zone.

ABM usage ensures a substantial prevalence of C-kit+ cells compared with the control group.

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Authors contribution

Lebedeva A.I. – collection and processing of material, carrying out of morphological studies, drafting of the article; Afanasiev S.A. – analysis and interpretation of data; final approval of the manuscript for publication; Muslimov S.A., Popov S.V. – conception and design of the study; Gareev E.M. – statistical processing of the obtained data; Kondratieva D.S. – carrying out of the main stages of the experiment.

Authors information

Lebedeva Anna I., Dr. Sci. (Biology), PhD, Senior Research Assistant, Department of Morphology, Russian Eye and Plastic Surgery Centre, Ufa. ORCID 0000-0002-9170-2600.

Muslimov Sagit A., Dr. Sci. (Med.), Leading Researcher, Department of Morphology, Russian Eye and Plastic Surgery Centre, Ufa. ORCID 0000-0002-9076-0251.

Gareev Evgeniy M., Cand. Sci. (Biology), Associate Professor, Senior Research Assistant, Russian Eye and Plastic Surgery Centre, Ufa. ORCID 0000-0002-6561-0892.

Afanasiev Sergey A., Dr. Sci.(Med.), Professor, Head of the Laboratory of Molecular Cell Pathology and Gene Diagnostics, Research Institute of Cardiology, Tomsk National Research Medical Centre, Russian Federation, Tomsk. ORCID 0000-0001-6066-3998.

Kondratieva Dina S., Cand. Sci. (Biology), Research Officer, Laboratory of Molecular Cell Pathology and Gene Diagnostics, Research Institute of Cardiology, Tomsk National Research Medical Centre, Russian Federation, Tomsk. ORCID 0000-0002-4004-2497.

Popov Sergey V., Dr. Sci. (Med.), Professor, Academician of RAS, Director of Research Institute of Cardiology, Tomsk National Research Medical Centre, Russian Federation, Tomsk. ORCID 0000-0002-9050-4493.

(✉) **Lebedeva Anna I.**, e-mail: Jeol02@mail.ru

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