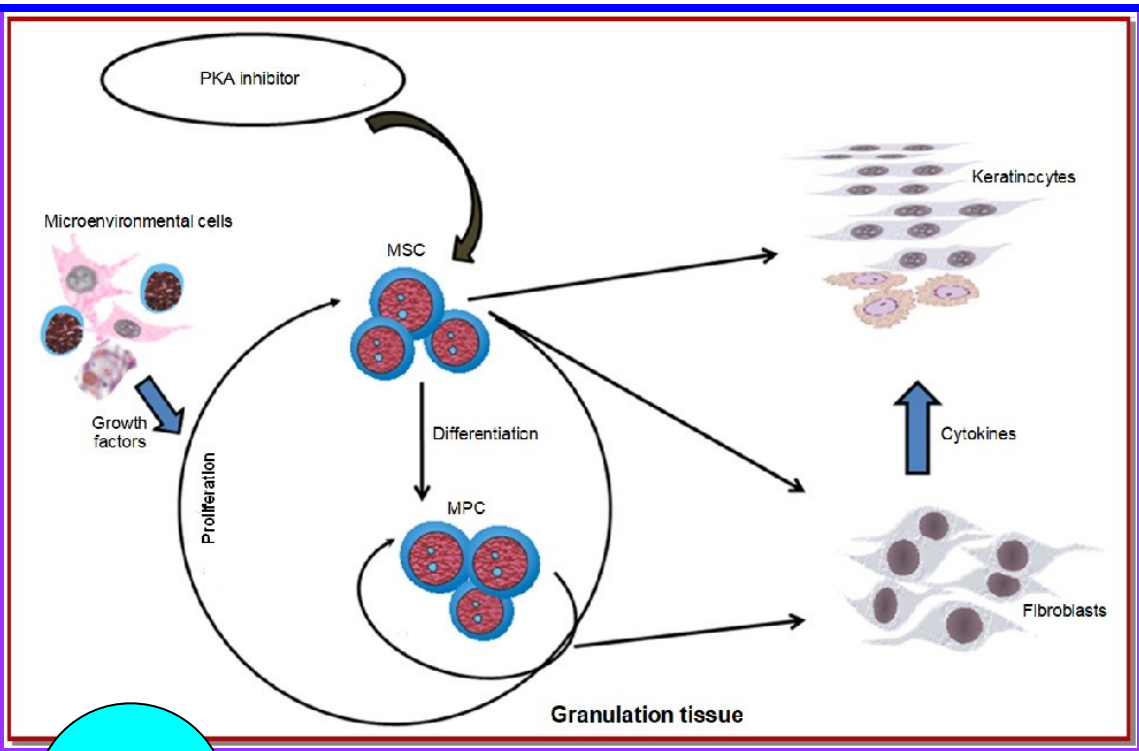

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Wound healing properties of the protein kinase A inhibitor and the mechanisms of their development

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Abstract

The regenerative activity of the protein kinase A inhibitor was investigated using externally on the model of the flap skin wound. The pronounced wound healing effects of the protein kinase A inhibitor had been revealed. They are based on the activation of mesenchymal progenitor cells. The development of this phenomenon was associated with the direct influence of the protein kinase A inhibitor on mesenchymal progenitor cells. The most significant stimulation of their growth potential occurred in the context of the impact of growth factors in particular fibroblast growth factor secreted by the stromal cells. Moreover, *in situ*, there was an increase not only in proliferating activity but also in the intensity of the specialization processes of progenitors. Without cytokines stimulation, the change in the pattern of cellular cAMP-mediated signal does not affect the maturation rate of precursors.

Introduction

There is a wide arsenal of wound healing drugs with different mechanisms of action. Some of them are a substrate for the formation of certain cell structures: nucleic acids and nucleotides (Liu et al., 2019), amino acids (Khalin et al., 2013), essential fatty acids (Silva et al., 2018), etc. These pharmacological substances stimulate cell proliferation in the damage zone by accelerating the synthesis of purine and pyrimidine bases, proteins, and other structural components of the cell. There are also tools based on biochemical process regulators (vitamins, trace elements) (Najeeb et al., 2016). Also, complex animal and plant-based drugs are suggested to heal wounds (Pereira et al., 2016; Firdous and Sautya, 2018; Ötün and Yücel, 2019). However, existing drugs are in some cases low-efficiency (Wang et al., 2019). Actively developed products based on the regulators of the processes of skin regeneration of protein origin: enzymes (Shah et al., 2018), proteins intercellular matrix (Wiser et al., 2019), growth factors, migration, stimulation of vasculogenesis, and other cytokines (Zarei et al., 2018, Wang et al., 2018). However, the protein nature of these substances predetermines their immunogenicity and high risk of allergic reactions (Khan et al., 2016). Therefore, the development of highly effective wound healing drugs with fundamentally new mechanisms is relevant.

The existence of numerous peculiarities in intracellular signalling in the regulation of the functions of progenitor cells of different types allowed to propose the development of a new direction of pharmacotherapy in regenerative medicine - Strategy of targeted pharmacological regulation of intracellular signal transduction in regenerative cells (Zyuz'kov et al., 2019a; Zyuz'kov et al., 2021).

It is assumed that the selectivity of stimulation of regeneration of the organs and tissues in need of this will be determined by the specific role of certain signalling molecules (Manning et al., 2002; Hankenson

et al., 2015; Zyuz'kov et al., 2019b) in the realization of the growth potential of progenitors against the background of tissue specificity of their different types and isoforms (including alternative splicing products) (Mavers et al., 2009; Mu et al., 2010; Durandy et al., 2020).

It is known that one of the key roles in the regulation of proliferation and differentiation of the progenitor cells, as well as in the secretion of cells microenvironment of tissues of growth factors plays cAMP-mediated signalling (Koga et al., 2019, Smith et al., 2019). However, new evidence has recently been obtained showing much more complex signal transduction through cAMP than previously thought. The implementation of effects involving this second messenger can take place not only through its interaction with the protein kinase A and in the further activation of CREB, but also through the activation of Ca2+/calmodulin-dependent protein kinase and changes in the pattern of regulation of MARK-pathways (Dhalla et al., 2010; Chen et al., 2016), or the phosphorylation of Epac (exchange protein directly activated by cAMP) (Cheng et al., 2008; Ahmed et al., 2019; Formoso et al., 2020), etc. Previous studies of the role of cAMP-mediated signalling in the regulation of the functions of different types of progenitors have revealed some ambiguous phenomena (Zyuz'kov et al., 2014; Schuller et al., 2019). It was concluded that to effectively manage the regulatory processes by modulating the cAMP-pathways, a targeted effect on the molecules responsible for certain directions of signal transduction is necessary.

A convenient model for the development of new approaches to solving the problems of regenerative medicine is the skin wound. Besides, the creation of fundamentally new wound healing facilities remains relevant. These products should not only speed up the process of tissue repair but also lead to the formation of full-fledged skin (Dehkordi et al., 2019). At the same time, it is believed that achieving such a result is possible due to the pharmacological activation of resident progenitor cells functions of the skin and the underlying tissues (Driskell et al., 2013; Motegi et al., 2017), as well as the mobilization and migration of multipotent stem cells (SC) from their tissue-depots (primarily from bone marrow) (Goldberg et al., 2006; Mishra et al., 2017; Fu et al., 2019).

The work aimed to study the wound healing effects of the protein kinase A inhibitor, and the mechanisms of their development associated with the functioning of mesenchymal progenitors.

Materials and Methods

Animals

Experiments were carried out on C57B1/6 mice (n= 119) at the age of 2-2.5 months, weighing 20-22 g. Animals of the 1st category (conventional outbred and linear mice) were obtained from the Experimental Biological Models Department of Goldberg Research Institute of Pharmacology and Regenerative Medicine (Russia) (certificate available). Before the beginning of experiments (during 10 days) and over the study period, animals were contained in the vivarium (air temperature 20–22°C, humidity 50-60%) in plastic cages (10-15 mice) on a normal diet, solid diet pellets (Limited

Box 1: Skin wound healing activity

Principle

The surgical mouse model of skin excisional wound healing used to study the cellular and molecular pathways involved in wound repair and regeneration.

Requirements

Alcohol (70%); Ether anesthesia; Eosin; Hair removal machine; Hematoxylin; Paraffin; protein kinase A inhibitor (KT3761) (Sigma-Aldrich, USA); Mice; Neutral formalin (10%); Scale; Stencil paint

Procedure

Step 1: To do this, on the depilated area of the back in mice under light ether anesthesia cut a flap of skin (diameter 10 mm).

Step 2: For longer healing of the scab from the wound regularly (through 24 hours) removed.

Step 3: The protein kinase A inhibitor was applied to the wound of the experimental mice (n=52) from the first day after the modelling of the wound, daily throughout the healing period of 20 µL (at a concentration of 10 µM).

Step 4: Control animals (n=52) were applied to the wound by the equivalent volume.

Step 5: The criteria for early healing were the average diameter of the wound (control and experienced group: n=20/20)

Step 6: The results of the histological study of the biopsies of the skin of mice obtained on day 3 and 5 of the wound defect (control and experienced group: n=12/12).

Step 7: The histological preparations of the skin (preparations were fixed in 10% neutral formalin, dehydrated in a series of alcohols with rising concentration, impregnated with paraffin, and cut into pieces of 4-5 microns thick) were stained with hematoxylin and eosin.

Step 8: The study of mesenchymal progenitors content in the wound, their functional activity, and production of growth factors by stromal cells from the surface of the wound.

Reference

Goldberg et al., 2006; Zyuz'kov et al., 2012

Reference (video)

Öz et al., 2016; Qureshi et al, 2019

Liability Company Assortiment Firm, Sergiev Posad city, Russia), water *ad libitum*. To exclude seasonal fluctuations of studied parameters, all the experiments were performed in the autumn-winter period. The animals were removed from the experiment (sacrificed).

Study of mesenchymal progenitors content in the wound and their functional activity

Studies of functional activity of mesenchymal progenitors in the wound were conducted on day 3 and day 5 of experience (control and experienced group: n= 20/20). Cells obtained after scraping from the wound surface in the concentration of 10^5/mL were incubated in StemMACS™ MSC Expansion Media (Miltenyi Biotec, Germany) for 7 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After incubation, the content of clonogenic cells, their proliferative activity, and the intensity of specialization (differentiation/ maturation) were calculated. The number of MPC was determined by the yield in the respective cultures of fibroblast colony-forming units (CFU-F, colonies containing more than 50 cells). The proliferative activity of the progenitor cells by the method of cell suicide using hydroxyurea (1 μM; Calbiochem, USA). The pool of CFU in the S-phase of the cell cycle was determined according to the formula: N = [(a-b)/a] × 100%, where a is the average for the group the number of CFU-F from cells not treated with hydroxyurea; b - the average for the group the number of CFU-F from cells treated with hydroxyurea. The intensity of the processes of specialization of progenitors was determined by calculating the ratio of the corresponding cluster-forming (ClFU-F, clusters containing 20-30 cells) to CFU -F (differentiation index) (Goldberg et al., 2006, Zyuz'kov et al., 2012).

Production of growth factors by stromal cells from the surface of the wound

Using cultural methods we studied the production of growth factors that stimulate the growth of CFU-F (colony-stimulating activity - CSA) by stromal cells scraped from the surface of the wound. To do this, adherent cells obtained after scraping from the wound surface in concentrations of 2 × 10^6 / ml were incubated in StemMACS™ MSC expansion media for 2 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After that, we received the conditioned media and determined their effect on the growth of CFU-F in the test system, which is a culture of bone marrow cells at a concentration of 10^5/mL (Zyuz'kov et al., 2012).

Effect on functioning of progenitors in vitro

The bone marrow of C57B1/6 mice (n=15) was used as a source of mesenchymal progenitors. Using the cultural methods, we studied the direct effect of the protein kinase A inhibitor on the realization of the growth potential of MPC *in vitro*. Bone marrow cells of concentration of 10^5/mL were incubated in StemMACS™ MSC expansion media for 7 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After incubation, the content of CFU-F, their proliferative activity, and the intensity of specialization was calculated as described above.

Changes in these parameters under the influence of the protein kinase A inhibitor (at a concentration of 10 μM) were investigated in cell incubation in the StemMACS™ MSC expansion media environment without fibroblast growth factor (FGF-basic, Sigma-Aldrich, USA) and when the main 20 ng FGF-basic was added to the medium.

Secretion of growth factors by stromal cells in vitro

Bone marrow cells in a concentration of 2 × 10^6/mL incubated in StemMACS™ MSC expansion media for 2 hours in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. Then adherent cells incubated in StemMACS™ MSC expansion media containing 10 μM the protein kinase A inhibitor for 2 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After that, we received the conditioned media and determined their effect on the growth of CFU-F in the test system (Zyuz'kov et al., 2012).

Statistical Analysis

The results were analyzed with one-way ANOVA followed by the Mann–Whitney test for independent samples. The data are expressed as arithmetic means (and standard errors in a table). The significance level was $p<0.05$.

Results

Wound healing effects

In the controls, the wounds healed by day 18 of the experiment. External use of the protein kinase A inhibitor led to a significant reduction in the period of repair of the tissue defect. By 14 a day there was a complete regeneration of the skin (Table I). At the same time, there was a decrease in the size of wounds for all observation periods, starting with the 3rd day of the experiment. The average wound diameter in mice that were treated at the wound of the protein kinase A inhibitor was 12.2, 12.4, 20.8, 17.2, 39.3, and 100% smaller than control animals on day 3, 5, 7, 9, 12, and 14, respectively).

In a histological study on day 3 after modeling the skin wound in control and experimental groups necrotic layer on the surface of the wound contained fibrin, under which there was a layer of granular tissue many cells (mostly neutrophils and macrophages). The inflammatory process (interstitial swelling and leukocyte infiltration) extended to the underlying layer of striated muscles. At the edges of the wound were swelling, dermis hyperemia, and overgrowth of the

Table I

Effects of protein kinase A inhibitor on the dynamics of skin wound healing in C57BL/6 mice

	Wound diameter (cm)	
Day	Control	PKA inhibitor
1	1.1 ± 0.0	1.1 ± 0.0
3	1.0 ± 0.0	0.9 ± 0.0[a]
5	0.9 ± 0.0	0.8 ± 0.0[a]
7	0.8 ± 0.0	0.6 ± 0.0[a]
9	0.6 ± 0.0	0.5 ± 0.0[a]
12	0.3 ± 0.0	0.2 ± 0.0[a]
14	0.1 ± 0.0	0.0 ± 0.0[a]
16	0.1 ± 0.0	0.0 ± 0.0[a]

Data are mean ± SEM; [a]Superscript means p<005 in comparison to control

epidermis, consisting of 8-10 layers of undifferentiated cells. On day 5, the newly formed epithelium at the edges of the wound was a layer of isomorphic cells. In the group of mice after the external use of the protein kinase A inhibitor, leukocyte infiltration of the edges of the wound, dermis, and underlying tissues on day 3 of experience was significantly lower. On day 5 of the experiment in this group of animals, there was a significant increase in the number of fibroblasts in the granulation tissue (Figure 1).

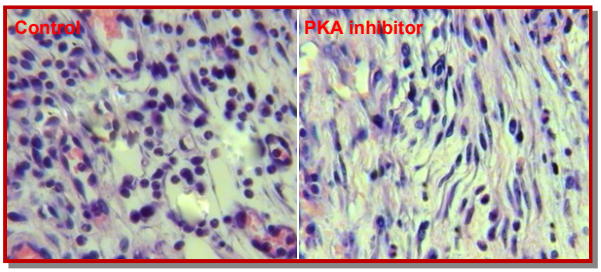

Figure 1: Effects of the PKA inhibitor on skin wound granulation tissue in C57BL/6 mice on day 5 after wound creation (hematoxylin and eosin staining; magnification x400)

These morphological findings were not only a criterion for accelerating tissue repair processes but also a sign of better skin regeneration (tissue restitution) in the future (Motegi et al., 2017, Mishra et al., 2017).

Content and functional activity of mesenchymal progenitors in the wound

The study of the mechanisms of wound healing action of the protein kinase A inhibitor revealed its pronounced effect on mesenchymal progenitors in the wound. There was a significant increase in the content of CFU-F in the wound (up to 1134.3 and 315.5% of

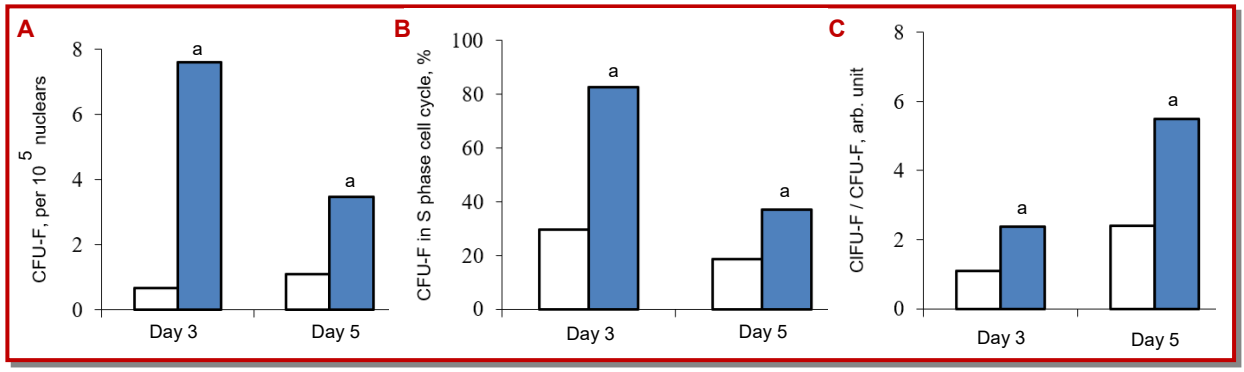

Figure 2: Effects of the PKA inhibitor on the number of CFU-F in wound surface (A), proliferative activity (B), and these cells differentiation index (C) in control C57BL/6 mice (white bars) and the treatment of the wound with the PKA inhibitor (blue bars). [a]p<0.05 in comparison with the control

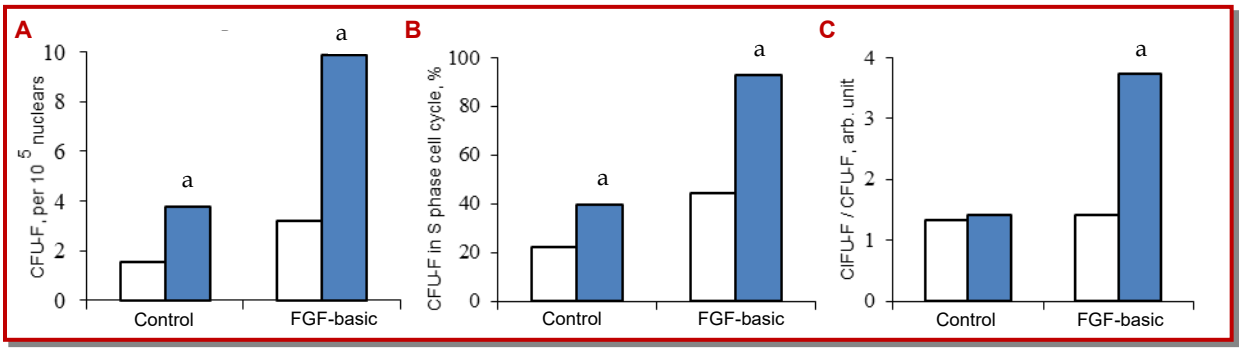

Figure 3: Effects of the PKA inhibitor on the number of CFU-F in wound surface (A), proliferative activity (B) and these cells differentiation index (C) in the cell culture of the bone marrow without FGF-basic (control) and with FGF-basic without signaling molecule inhibitor (white bars) and when the inhibitor (blue bars) are added to the medium; Used the Mann-Whitney test ([a]p<0.05 in comparison with the control)

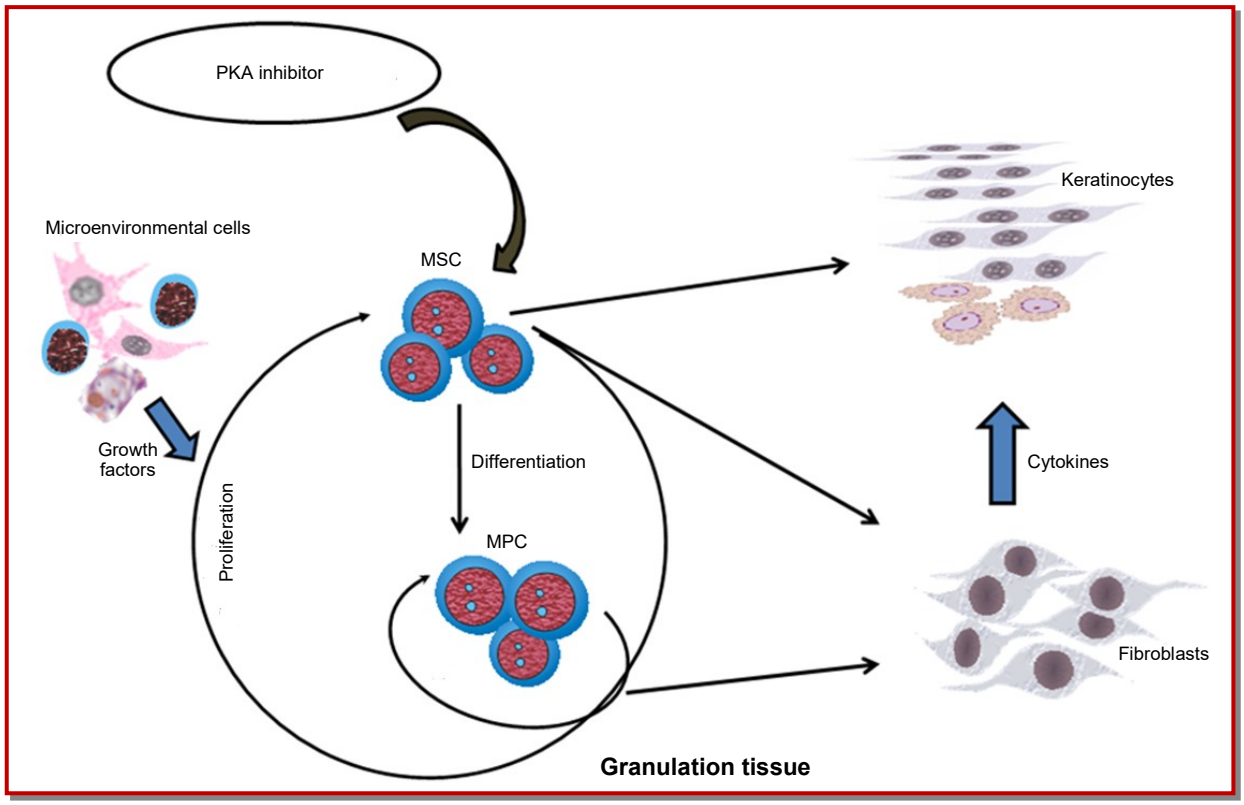

Figure 4: Mechanisms of wound healing action of protein kinase A inhibitor. MSC - multipotent mesenchymal stem cells, MPC – committed mesenchymal progenitor cells

control at day 3 and 5 respectively), their proliferative activity (up to 279.4 and 198.4% of control at day 3 and 5 respectively), and the intensity of differentiation (up to 216.4 and 229.2% of control at day 3 and 5 respectively) (Figure 2).

Secretion of growth factors by stromal wound cells

The experiments did not detect changes in the secretion of growth factors by stromal cells obtained from the surface of the wound. The level of the conditioned media CSA of these cells in animals of the experimental group did not differ from that of control mice (data not shown).

Functioning of progenitors in vitro

The addition of the protein kinase A inhibitor to the culture of bone marrow cells increased the number of CFU-F and their mitotic activity (to 248.7 and 206.7% of control (media without protein kinase A inhibitor) respectively). There was no change in the intensity of the specialization processes of mesenchymal predecessors (Figure 3).

Studies of these parameters using the cultural environment with FGF-basic have revealed slightly different phenomena. In this case, there was not only an increase in the number of CFU-F and their number in the S-phase of the cell cycle but also a significant increase in the intensity of maturation of progenitors (up to 263.8% of the similar parameter in the media with FGF-basic without the protein kinase A inhibitor). Moreover, these changes look particularly interesting given that the FGF-basic without the protein kinase A inhibitor did not affect the specialization processes of mesenchymal precursors. Also, the increase in proliferative activity of CFU-F when the protein kinase A inhibitor was added in the media with FGF-basic compared to the value of this indicator without the protein kinase A inhibitor was 208.5%. This was higher than the increase in the mitosis rate of mesenchymal progenitors under the influence of the protein kinase A inhibitor in the media without FGF-basic (Figure 3).

Secretion of growth factors by stromal cells in vitro

The introduction of the protein kinase A inhibitor *in vitro* into the culture of bone marrow cells did not affect the formation of the level of the CSA conditioned media. The values of this parameter were 4.52±0.31 and 4.49±0.27 arbitrary units from supernatants from cells cultivated in the media without the protein kinase A inhibitor and with this inhibitor, respectively.

Discussion

The results indicate the presence of pronounced wound healing properties in the cyclic AMP-dependent protein kinase A inhibitor in its external application. It was

found that the implementation of the identified pharmacological effects of this substance is its direct effect on the progenitors in the wound (Figure 4). Moreover, the most significant increase in their functional activity occurs if they are influenced by growth factors (in particular FGF (Yang et al., 2018) secreted by the stromal cells of the microenvironment (as well as, probably, migrating to the wound immunocompetent cells (Ali et al., 2017; Jiang et al., 2020)). In this case, *in situ*, there is an increase in both proliferation activity and the intensity of progenitor specialization processes. Without this cytokine stimulation, the change in the pattern of cellular cAMP-mediated signaling does not affect the maturation rate of fibroblast precursors playing one of the key roles in skin reparation (Driskell et al., 2013, Dehkordi et al., 2019). However, the experiments have shown that the "basic" level of production of growth factors (since the blockage of protein kinase A in the cells of the microenvironment of granular tissue did not affect their secretory function) is sufficient to implement the described mechanism.

At the same time, the progenitor cells pool, participating in this case in skin regeneration is represented by the cells of the basal layer of the skin located near the site of the injury (Dehkordi et al., 2019; Yang et al., 2020), resident committed mesenchymal precursors of nearby tissues (Driskell et al., 2013, Motegi et al., 2017), as well as multipotent SC, mobilized from the tissue-depots, primarily bone marrow, and migrated to the skin wound (Goldberg et al., 2006; Mishra et al., 2017; Fu et al., 2019). Therefore, the development of therapeutic approaches with protein kinase A inhibitors is consistent with the principles of carcinogenic drug safety (James et al., 2009 Drelon et al., 2016). This criterion is an inalienable factor for the creation of drugs for regenerative medicine (in terms of minimizing the potential risks of tumor transformation of progenitors while stimulating their proliferative activity).

Conclusion

The use of protein kinase A inhibitors (as well as possibly CREB inhibitors) in skin wounds is a promising approach to skin repair.

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Ethical Issue

The study was approved by the Institute`s local Ethics Committee (Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences. All animal experiments were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Conflict of Interest

Authors declare no conflict of interest

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